No. 90 VOLUME XXII SEPTEMBER 1961

American Journal of

Veterinary RESEARCH

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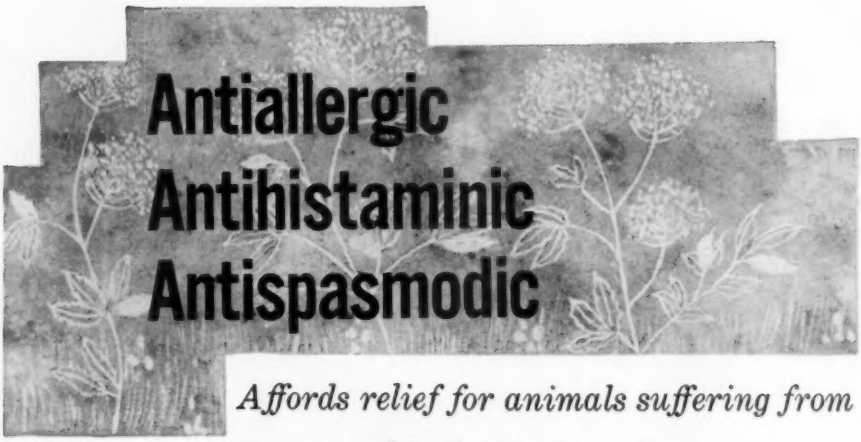

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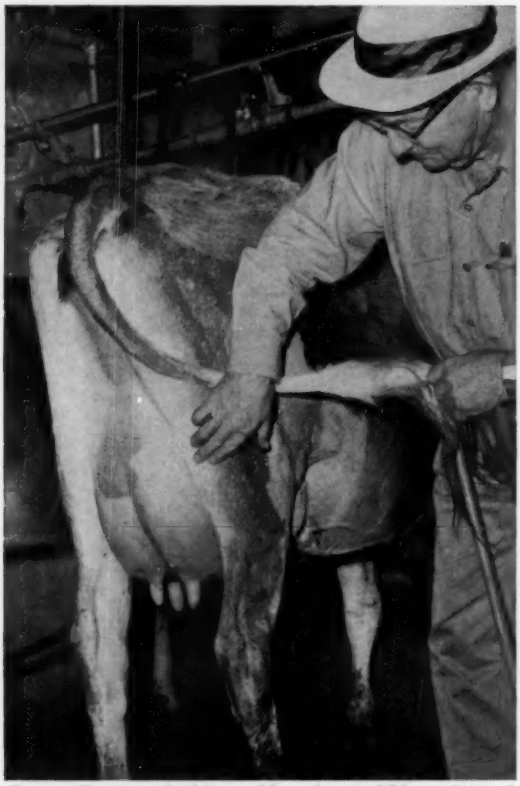

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VOL. XXII SEPTEMBER, 1961 No. 90

The Ages at Which the Centers of Ossification Appear Roentgenographically in the Limb Bones of the Dog

W. C. D. Hare, B.Sc., Ph.D., D.V.M., D.V.S., M.R.C.V.S.

SUMMARY

Age periods for the appearance of the ossification centers that appear after birth in the limb bones of the dog were determined by roentgenography, at intervals from birth, of dogs from 4 breeds: namely, the German Shepherd Dog, the Collie, the Bulldog, and the Beagle. Twenty-four dogs were examined through the first 3 weeks of their lives, and 14 of these were examined through the next 5 weeks of their lives.

The ossification centers appeared in approximately the same chronologic order, but the ages at which they appeared were subject to variation. The variations occurred between littermates and individuals of the same breed, and, to a greater extent, between individuals of different breeds. The sex of the animal did not appear to influence the ages at which the centers appear.

INTRODUCTION

The ages at which the ossification centers appear in the limb bones of the dog have been studied by Retterer,[18] Touissant [23] (Fleming [5]), Lesbre,[10] Schaeffer,[19] Pomriaskinsky-Kobozieff and Kobozieff,[13] and Bressou *et al.*[2]

The earlier workers (Retterer,[19] Touissant,[23] and Lesbre [10]) provided information that was general because they had to rely on dissection and maceration, which are not the best means of determining when the centers appear since, in the beginning, the centers are extremely small and hard to find. Schaeffer,[19] however, first stained the bones with alizarin red and then cleared them. This method gives very accurate results, but it necessitates killing the dogs and, therefore, requires a large number of animals in order to make a comprehensive study. For this reason, Schaeffer's findings are of limited value because he had only a small number of dogs available for study. Pomriaskinsky-Kobozieff and Kobozieff [13] and Bressou *et al.*[2] made roentgenographic studies of the ossification process in the manus and pes, respectively. Roentgenography has an advantage over other methods because it allows the ossification process to be followed throughout the entire growth period of each animal.

This study was undertaken to determine when the ossification centers appear in the limb bones proximal to the manus and pes, and to confirm when they appear in the manus and pes, since the previous studies by Pomriaskinsky-Kobozieff and Kobozieff [13] and Bressou *et al.*[2] were made on more limited numbers of dogs.

Materials and Methods

The study was carried out on dogs* of 4 breeds: the German Shepherd Dog, the Collie, the Bulldog,

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From the Laboratories of Anatomy, the School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

Based on part of a thesis submitted to the University of Edinburgh for the degree of Doctor of Veterinary Medicine and Surgery.

The author acknowledges the support received from Dr. D. G. Lee, the School of Veterinary Medicine, University of Pennsylvania, and Dr. J. H. Ballantyne, Ontario Veterinary College, Canada. He also thanks Dr. J. E. Croshaw, Jr., for reading the manuscript and Mr. A. Marfaing, Wistar Institute, for taking the photographs.

* Dogs supplied through the generosity of dog breeders in Ontario, Canada.

and the Beagle. With the exception of the Collies, which came from 2 litters, the dogs of each breed were from the same litter. Roentgenographs of all the dogs were taken at intervals, starting soon after birth. The number of dogs examined, their sex, and the ages at which they were roentgenographed are given (Table 1). Twenty-four dogs, of nearly even sex distribution, were examined through the first 3 weeks of life, and 14 dogs, of even sex distribution, were examined through the first 8 weeks of life.

Prior to roentgenography, the dogs were anesthetized with a barbiturate. At each examination, an attempt was made to obtain 2 views of each limb: a lateral and a dorsovolar (dorsoplantar).

The care and management of the dogs has been described in an earlier paper.[7] The dogs were housed in kennels with outside runs. The pups were given milk and cod liver oil daily. After they had been weaned, the dogs were fed canned dog food supplemented with minerals and vitamins whenever necessary. Regular worm egg counts were made, and treatment was given if required. The pups were given regular doses of canine anti-distemper serum up to the age of 3 months, at which time they were vaccinated against canine distemper and infectious canine hepatitis. Rabies vaccine was also administered to all the dogs.

The humeri from 3 dogs, 5 months old, were specially stained to determine whether there was a separate center of ossification for the tuberculum majus. The method used was that of Haviland.[8] The bones were immersed in a 2% solution of potassium hydroxide until the tissues were clear. Then a saturated solution of alizarin red S in 95% alcohol was added to the 2% solution of potassium hydroxide until the latter turned a wine color. The bones were left in this solution until they were stained, and then they were decolorized by immersion in a solution consisting of equal parts of a 10% solution of glycerin and a 2% solution of potassium hydroxide. After decolorization, the bones were cleared by passing them through ascending grades of glycerin (25%, 50%, 75% and 100%).

TABLE 1—The Number of Dogs Examined, Their Sexes, and Ages at the Time of Roentgenography

German Shepherd Dog		Collie (No. 1 litter)		Collie (No. 2 litter)		Bulldog		Beagle	
Days	No. & Sex	Days	No. & Sex	Days	No. & Sex	Days	No. & Sex	Days	No. & Sex
3	3M, 4F	3	3M, 4F	3	2M, 2F	0	1M, 1F	3	4M, 2F
6	3M, 4F	7	3M, 4F	4	1M, 1F	2	1M, 1F	7	4M, 2F
10	3M, 4F	11	3M, 3F	5	1M, 1F	4	1M, 1F	11	4M, 2F
15	3M, 4F	17	3M, 3F	6	1M, 1F	8	1M	14	4M, 2F
22	3M, 4F	21	3M, 3F	7	1M, 1F	9	1M	21	4M, 2F
30	3M, 4F	26	3M, 3F	8	1M, 1F	10	2M	28	2M, 2F
46	2M, 3F	34	2M, 3F	9	1M, 1F	11	2M	42	2M, 2F
65	2M, 2F	40	2M, 3F	10	1F	15	2M	60	2M, 2F
		55	2M, 3F	11	1M, 1F	17	2M		
				12	1F	19	2M		
				13	1M, 1F	25	1M		
				14	1F	31	1M		
				17	1M, 2F	38	1M		
				19	1M, 2F	45	1M		
				21	1M, 2F	52	1M		
				23	1M, 2F	59	1M		
				25	1M, 2F				
				28	1M, 2F				
				31	1M, 2F				

The No. 2 litter of Collies was divided into 2 groups: dogs in these groups were roentgenographed on alternate days from the 4th or 5th to the 17th day, and together from the 17th day on. The litter of Bulldogs was divided into 2 groups: the groups were roentgenographed on alternate days to the 9th day; thereafter, the surviving dogs from each group were roentgenographed together.

The criterion for the time of the appearance of an ossification center was taken to be the appearance of an opaque area on the roentgenogram.

Results

Scapula.—The scapula develops from 2 principal centers of ossification: one for the corpus scapulae, which is present and well developed at birth; and one for the tuber scapulae, which appears during the 5th, 6th, 7th, or 8th week after birth, depending on the breed (Table 2).

Humerus.—The humerus develops from 5 principal centers of ossification: one for the corpus humeri (diaphysis); one for the caput humeri, tuberculum majus, and tuberculum minus (epiphysis proximalis); one for the capitulum et pars lateralis trochleae; one for the pars medialis trochleae; and one for the epicondylus medialis.

The center for the diaphysis is present and well developed at birth. The center for the epiphysis proximalis (Fig. 1) appears during the 1st or 2nd week of life (Table 2). The writer was unable to confirm that there is a separate center for the tuberculum majus as described by Lesbre,[10] Ellenberger

and Baum,[4] and Seoudi.[20] The centers for the capitulum et pars lateralis trochleae and for the pars medialis trochleae (Fig. 1) appear during the 2nd or the 3rd week of life, but the center for the epicondylus medialis (Fig. 5) does not appear until the 5th, 6th, 7th, or 8th week of life (Table 2).

Radius.—The radius develops from 3 principal centers of ossification: one for the corpus radii (diaphysis) and one each for the caput radii (epiphysis proximalis) and the trochlea et processus styloideus radii (epiphysis distalis).

The center for the diaphysis is present and well developed at birth. The center for the epiphysis proximalis (Fig. 5) appears during the 3rd, 4th, or 5th week and one for the epiphysis distalis (Fig. 3) a little earlier, during the 2nd, 3rd, or 4th week (Table 2).

Ulna.—The ulna develops from 3 principal centers of ossification: one for the olecranon et corpus ulnae (diaphysis); one for the pars caudalis tuberis olecrani (epiphysis proximalis); and one for the caput ulnae (epiphysis distalis). In 2 dogs (Table 2), the epiphysis proximalis developed from 2 small centers, which united rapidly to form the principal center; in one dog (Table 2), the epiphysis distalis developed from 2 small centers, which likewise united rapidly to form the principal center.

The center for the diaphysis is present and well developed at birth. The centers for the epiphysis proximalis (Fig. 5) and the epiphysis distalis appear during the 2nd month of life; however, the center for the epiphysis distalis usually appears first and, as a result, is better developed at the end of the age period (Table 2). The Bulldog was an exception since, in this dog, at

Fig. 1—Mediolateral view of the right brachium of a 10-day-old female German Shepherd Dog. Centers of ossification for (a) the epiphysis proximalis humeri, and (b) the capitulum et pars lateralis trochleae and the pars medialis trochleae.

Fig. 2—Right pelvic limb of a 13-day-old male Collie dog. Centers of ossification for (a) the epiphysis distalis femoris, (b) the condylus medialis et condylus lateralis tibiae, (c) the os tarsi fibulare, (d) the os tarsale quartum, (e) the caput femoris, (f) the os tarsi tibiale, and (g) the os tarsi centrale.

TABLE 2—The Ages at Which Centers of Ossification Appeared Roentgenographically

Center	German Shepherd Dog	Collie	Bulldog	Beagle
SCAPULA				
Corpus scapulae*				
Tuber scapulae	30–46d (5)	40–55d (5)	52d (1)	42–60d (4)
HUMERUS				
Diaphysis*				
Epiphysis proximalis	3–6d (7)	3–5d, 4–6d (1) (2) 5–7d, 3–7d (1) (7)	8d, 9d (1) (1)	7–11d (6)
Capit. et pars lat. trochleae				
Pars medialis trochleae	6–10d (7)	10d, 11d, 13d (1) (2) (1) 11–17d (6)	15d, 17d (1) (1)	14d, 14–21d (1) (5)
Epicondylus medialis	30–46d (5)	40–55d (5)	52–59d (1)	42–60d (4)
RADIUS				
Diaphysis*				
Epiphysis proximalis	15–22d, 22–30d (4) (3)	21d, 23d, 25d (1) (2) (4) 21–26d (5)	31–38d (1)	21–28d (4)
Epiphysis distalis	10–15d, 15–22d (5) (2)	11–17d, 13–17d (2) (2) 17–19d, 17–21d (1) (4)	25d (1)	14–21d, 21–28d (1) (3)
ULNA				
Diaphysis*				
Epiphysis proximalis	30–46d (5) 2 dogs, 2 centers	40–55d (5)	52–59d (1)	42–60d (4)
Epiphysis distalis	30–46d (5) 1 dog, 2 centers	40–55d (5)	59d (1)	42–60d (4)
CARPUS				
Os carpi ulnare	22–30d (7)	21–26d, 26–34d (2) (3) 25d, 25–28d (1) (2)	59–68d (1)	28–42d (4)
Os carpi intermedium	10–15d (7)	17–19d, 19–21d (2) (1) 17–21d, 21–26d (2) (4)	31–38d (1)	21d, 21–28d (1) (3)
Os carpi radiale	15–22d, 23–30d (2) (5)	25d, 25–28d (1) (2) 26d, 26–34d (2) (3)	38–45d (1)	28–42d (4)
Os carpi centrale	22–30d, 30d (5) (5)	31d, 26–34d (2) (5)	59–68d (1)	42–60d (4)
Carpus oss. acc.	6–10d (7)	8d, 9d (2) (2) 7–11d (6)	17d (2)	11–14d, 14–21d (2) (2) 21–28d (2)
Tub. oss. acc.	30–46d, 46–65d (4) (1)	40–55d (5)	54–68d (1)	42–60d (4)
Os. carp. primum	15–22d (7)	19d, 21d (3) (3) 21–26d (3)	31–38d (1)	21–28d, 28–42d (3) (1)
Os. carp. secundum	22–30d, 30–46d (3) (2)	23d, 25d (1) (1) 25-28d, 21–26d (1) (6)	52–59d (1)	28–42d (4)
Os. carp. tertium	22–30d (7)	23d, 25d (1) (2) 21–26d (6)	38–45d (1)	28–42d (4)
Os. carp. quartum	15–22d (7)	19d, 17–21d (3) (4) 21–26d (2)	28–31d (1)	21–28d, 28–42d (3) (1)
OS COXAE				
Corpus et ala ossis ilii*				
Ramus et ala ossis ischii*				
Corpus et rami ossis pubis*				

(continued on page 829)

TABLE 2 (continued)—The Ages at Which Centers of Ossification Appeared Roentgenographically

Center	German Shepherd Dog	Collie	Bulldog	Beagle
Os acetabulum	46–65d (4)	55–69d (5)	68–80d (1)	.....
Tuber ischiadicum	46–65d (4)	55–69d, 69–83d (2) (3)	80–94d (1)	.....
Crista iliaca	95–130d (3)	111–118d (5)	121–135d (1)	.....
Os interischiadicum	130–162d (3)	118–132d (5)	.....	.....
Arcus ischiadicus	160–203d (3)	158d (5)	.....	.....
FEMUR				
Diaphysis*				
Caput femoris	3–6d, 6–10d (2) (5)	3–7d, 7–11d, 5d (2) (4) (1) 6–8d, 7–9d (2) (1)	11–15d (2)	11–14d, 14–21d (2) (4)
Trochanter major	30–46d (5)	34–40d, 40–55d (2) (3)	52–59d (1)	60d (4)
Trochanter minor	46–65d (4)	40–55d (5)	68–80d (1)	66–69d (4)
Epiphysis distalis	10–15d (7)	7–11d, 11–17d, 11d (2) (4) (1) 11–13d, 12d (1) (1)	19–25d (1)	14–21d, 21–28d (3) (1)
TIBIA				
Diaphysis*				
Cond. med. et lat.	10–15d (7)	11–17d, 13d (6) (1) 13–17d, 17–19d (1) (1)	19–25d (1)	14–21d, 21–28d (1) (3)
Tuberositas tibiae	46d, 46–65d (4) (1)	40–55d (5)	68–80d (1)	42–60d (4)
Malleolus medialis	65–95d (3)	55–69d (5)	80–97d (1)	.....
Epiphysis distalis	10–15d (7)	11–17d, 13–17d (6) (1) 17–19d (2)	25–31d (1)	21–28d (4)
FIBULA				
Diaphysis*				
Epiphysis proximalis	30–46d (5)	34–55d, 40–55d (3) (2)	69–80d (1)	60–66d (4)
Epiphysis distalis	22–30d (7)	28–31d, 26–34d (3) (5)	42–52d (1)	28–42d (3)
TARSUS				
Os tarsi tibiale*				
Corpus et processus calcanei ossis tarsi fibulare*				
Tuber calcanei	30–46d, 46d (4) (1) 2 dogs, 2 centers	34d, 34–55d (1) (2) 40–55d (2)	52–59d (1)	42–60d (4)
Os tarsi centrale	10–15d (7)	11d, 13d, 14d (1) (1) (1) 11–17d (6)	31–38d (1)	11–14d, 14–21d (2) (3) 21–28d (1)
Os tarsale primum	30–46d (5)	25–28d, 28–31d (1) (2) 26–34d (5)	38–45d (1)	28–42d (4)
Os tarsale secundum	22–30d, 30–46d (6) (1)	25d, 25–28d (1) (2) 26–34d (5)	45–52d (1)	28–42d (4)
Os tarsale tertium	15–22d, 22–30d (2) (5)	21–23d, 21–26d (3) (6)	31–38d (1)	21–28d, 28–42d (3) (1)
Os tarsale quartum	7–10d, 10–15d (5) (2)	9d, 12d, 13d (1) (1) (1) 7–11d, 11–17d (2) (4)	19d (1)	11d, 11–14d (1) (4) 14–21d (1)
METACARPUS ET METATARSUS				
Diaphyses*				
Epiphyses mtc. II–V	22d, 22–30d (3) (4)	23–25d, 21–25d (3) (3) 26–34d (2)	31–38d (1)	28d, 28–42d (3) (1)

(continued on page 830)

TABLE 2 (continued)—The Ages at Which Centers of Ossification Appeared Roentgenographically

Center	German Shepherd Dog	Collie	Bulldog	Beagle
Epiphysis mtc. I	30–46d (5)	40–55d (5)	42–52d (1)	28–42d (4)
Epiphyses mtt. II–V	23–30d (7)	25–28d, 26–34d (3) (5)	45d (1)	28–42d (4)
Metatarsal I	30–46d, 46–65d (1) (3)	40–55d (5)	59d (1)	42–60d (4)
OSSA DIGITORUM MANUS ET PEDIS				
Epiphyses ph. prox. II–V pect.	23–30d (7)	21–26d, 26–34d (2) (3) 28d (3)	31–38d (1)	28–42d (4)
Epiphysis ph. prox. I pect.	30–46d (5)	40–55d (5)	52–59d (1)	42–60d (4)
Epiphyses ph. med. II–V pect.	30d, 30–46d (1) (4)	40–55d (5)	52–59d (1)	42–60d (4)
Epiphyses ph. prox. II–V pel.	22–30d (7)	26–34d, 28–31d (5) (3)	38–45d (1)	28–42d (4)
Epiphyses ph. med. II–V pel.	30–46d (5)	40–55d (5)	52–59d (1)	28–60d (4)
OSSA SESAMOIDEA				
Oss. sesamoid. vol.	46–65d (4)	55–69d (5)	94–108d (1)	66d, 69–75d (2) (2)
Oss. sesamoid. plant.	46–65d (3)	40–55d (5)	94–108d (1)	66d (4)
Oss. sesamoid. dors. pect.	109–130d, 95–130d (1) (2)	118d (5)	121–138d (1)	.....
Oss. sesmoid. dors. pel.	95–130d (3)	97–111d (5)	135–156d (1)	.....
Phacoid	95d (4)	97d, 97–118d (3) (2)	121–138d (1)	.....
Patella	30–46d, 46–65d (2) (2)	40–55d (5)	68d (1)	60d, 66d, 69d (1) (1) (2)
Fabellae	65–95d (4)	69–83d (5)	94–108d (1)	.....
Os sesamoid. popliteum	65–95d, 95d (2) (1)	83–97d (5)	135d (1)	.....

* Present at birth.
The number of dogs is shown in parentheses; d = day.

59 days, the center for the epiphysis proximalis was easily visible, whereas that for the epiphysis distalis was just appearing (Table 2).

Carpus.—The carpus develops from 10 centers of ossification. There is one center each for the os carpi ulnare, the os carpale primum, the os carpale secundum, the os carpale tertium, and the os carpale quartum. The os carpi accessorium develops from 2 centers, one for the corpus ossis accessorii and one for the tuberculum ossis accessorii (epiphysis). The so-called os carpi radiale represents the fusion of the os carpi radiale, the os carpi intermedium, and the os carpi centrale, and it develops from 3 centers, one for each of these bones.

The findings (Table 2) show that the ages at which the various centers of ossification appear in the carpus differ between breeds and, to a lesser extent, between individual animals within a breed. There may also be slight variations in the chronologic order of appearance. However, it can be stated, in general terms, that the center for the corpus ossis accessorii is the first to appear, usually during the 2nd week of life, and the other centers appear from the 3rd to the 9th week (Fig. 3), with the center for the tuberculum ossis accessorii the last to appear.

Os Coxae.—The os coxae develops from 7 or 8 centers of ossification. There is one center for the corpus et ala ossis ilii, one for the ramus et ala ossis ischii, one for the corpus et rami ossis pubis, one for the os acetabulum, one for the crista iliaca, and one for the tuber ischiadicum. The arcus ischiadicus develops from one or more centers and, in addition, there is a center for the small, wedge-shaped piece of bone, the os interischiadicum, which develops in the angle of divergence between the ossa ischii at the caudal end of the symphysis pelvis.

The centers for the corpus et ala ossis ilii, the ramus et ala ossis ischii, and the corpus et rami ossis pubis are present and well developed at birth. The center for the os

acetabulum appears during the 8th, 9th, 10th, or 11th week (Table 2). This center is difficult to distinguish roentgenographically, and it was not possible to determine the age at which it appears in the Beagle. The center for the tuber ischiadicum appears toward the end of the 2nd, or during the 3rd, month (Table 2). The center for the os interischiadicum appears during the 5th month, and the one or more centers for the arcus ischiadicus appear during the 6th month (Table 2).

Femur.—The femur develops from 5 principal centers of ossification: one for the corpus femoris (diaphysis); one for the caput femoris; one for the trochanter major; one for the trochanter minor; and one for the condylus medialis et condylus lateralis (epiphysis distalis).

The center for the diaphysis is present and well developed at birth. The center for the caput femoris (Fig. 2) appears during the 1st, 2nd, or 3rd week of life (Table 2). The center for the trochanter major appears during the 2nd month, and the one for the trochanter minor during the 7th, 8th, 9th, 10th, or 11th week (Table 2). The center for the epiphysis distalis (Fig. 2) appears during the 2nd, 3rd, or 4th week (Table 2).

Tibia.—The tibia develops from 5 principal centers of ossification: one for the corpus tibiae (diaphysis); one for the condylus medialis et condylus lateralis; one for the tuberositas tibiae; one for the malleolus medialis; and one for the extremitas tibiae (epiphysis distalis).

The center for the diaphysis is present and well developed at birth. The centers for the condylus medialis et condylus lateralis and for the epiphysis distalis (Fig. 2, 6) appear during the 2nd, 3rd, or 4th week (Table 2). The center for the tuberositas tibiae appears sometime from the 7th to the 11th week, depending on the breed (Table 2). The center for the malleolus medialis (Fig. 7) appears during the 3rd month.

Fibula.—The fibula develops from 3 principal centers of ossification: one for the corpus fibulae (diaphysis); one for the caput fibulae (epiphysis proximalis); and one for the malloleus lateralis (epiphysis distalis).

The center for the diaphysis is present and well developed at birth. The center for the epiphysis proximalis appears during the 2nd, or early in the 3rd, month (Table 2). The center for the epiphysis distalis appears during the 4th, 5th, 6th, or 7th week (Table 2). In 2 dogs, the distal epiphysis developed from 2 centers, which fused rapidly.

Tarsus.—The tarsus of the dog is composed of 7 bones. Each bone develops from a single center of ossification with the exception of the os tarsi fibulare, which develops from 2 centers, one for the corpus et processus calcanei and one for the tuber calcanei.

The centers for the corpus et processus calcanei ossis tarsi fibulare and the os tarsi tibiale appear before birth. The remaining centers appear after birth. The results of

Fig. 3—Mediolateral view of the right manus of a 28-day-old female Collie dog. Centers of ossification for (a) the os carpi intermedium, (b) the os carpale tertium, (c) the os carpale primum, (d) the epiphysis proximalis phalangis proximalis, (e) the epiphysis distalis radii, (f) the os carpi accessorium, (g) the os carpi ulnare, (h) the os carpale quartum, and (i) the epiphysis distalis ossis metacarpalis.

Fig. 4—Mediolateral view of the right pes of the 28-day-old female Collie shown in Figure 3. Notice that the epiphyses of the ossa metatarsalia and the phalanges proximales have not yet appeared as they have in the manus. Centers of ossification for (a) the os tarsi tibiale, (b) the os tarsi centrale, (c) the os tarsale tertium, (d) the epiphysis distalis tibiae, (e) the os tarsi fibulare, and (f) the os tarsale quartum.

the investigation (Table 2) indicate that in the tarsus, as in the carpus, the ages at which the centers appear vary from one breed to another and, to a lesser degree, between individual animals within a breed. There is, however, little variation in the chronologic order of their appearance. In all the breeds under study, the center for the os tarsale quartum is the 1st to appear after birth, during the 2nd or 3rd week, and the center for the os tarsi centrale is the 2nd to appear, during the 2nd, 3rd,

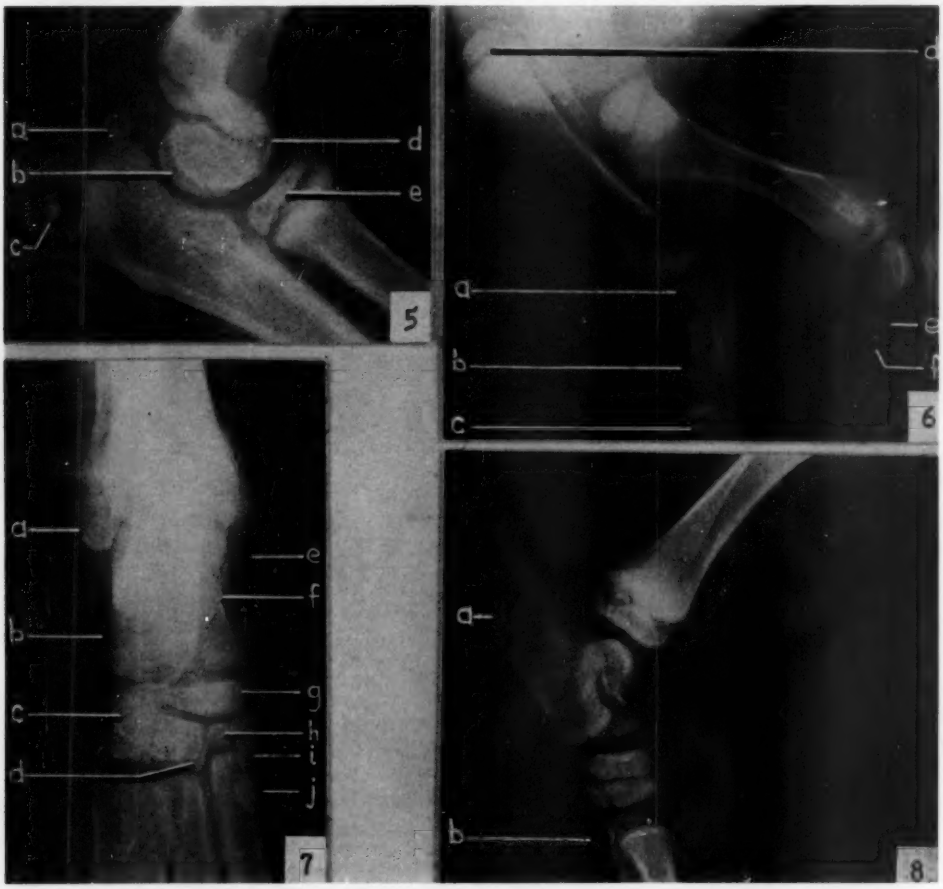

Fig. 5—Mediolateral view of the left articulatio cubiti of a 46-day-old female German Shepherd Dog. Centers of ossification for (a) the epicondylus medialis humeri, (b) the pars medialis trochleae, (c) the epiphysis proximalis ulnae, (d) the capitulum et pars lateralis trochleae, and (e) the epiphysis proximalis radii.

Fig. 6—Lateral view of the pelvic limbs of a 25-day-old female Collie Dog. Centers of ossification for (a) the epiphysis distalis tibiae, (b) the os tarsi tibiale, (c) the os tarsale quartum, (d) the epiphysis proximalis tibiae, (e) the os tarsi centrale, and (f) the os tarsale tertium.

Fig. 7—Plantarodorsal view of the left tarsus of a 94-day-old male Bulldog: (a) the epiphysis distalis fibulae, (b) the os tarsi fibulare, (c) the os tarsale quartum, (d) the os tarsale tertium, (e) the center of ossification for the malleolus medialis, (f) the os tarsi tibiale, (g) the os tarsi centrale, (h) the os tarsale secundum, (i) the os tarsale primum, and (j) the os metatarsale primum.

Fig. 8—Mediolateral view of the left tarsus of a 46-day-old female German Shepherd Dog: (a) 2 centers of ossification for the tuber calcanei, and (b) the center of ossification for the os metatarsale primum, just appearing.

4th, or 5th week (Fig. 2). The center for the os tarsale tertium (Fig. 6) appears next, followed by the centers for the os tarsale primum and the os tarsale secundum. The center for the tuber calcanei is the last to appear and, in 2 dogs, it developed from 2 small centers which fused rapidly (Fig. 8). By the end of the 2nd month, all the tarsal ossification centers have appeared.

Metacarpus et Metatarsus.—In the pectoral and pelvic limbs, the functional digits are the 2nd, the 3rd, the 4th, and the 5th. The ossa metacarpalia and the ossa metatarsalia that articulate with these digits are the most well developed. Each of these bones develops from 2 centers of ossification, one for the basis et corpus (diaphysis) and one for the caput (epiphysis distalis).

The centers for all the diaphyses are present and well developed at birth. The centers for the epiphyses distales do not all appear at the same time: the centers in the 3rd and 4th bones appear first, followed shortly by the center in the 2nd bone, and a little later by the center in the 5th bone (Table 2). The centers in the ossa metatarsalia appear a little later than the centers in the corresponding ossa metacarpalia (Fig. 3, 4).

The os metacarpale 1 is not as large as the other 4 ossa metacarpalia. It develops from 2 centers of ossification, but, in this case, one center is for the corpus et caput (diaphysis) and the other is for the basis (epiphysis proximalis). The center for the diaphysis is present at birth, while the center for the epiphysis proximalis appears during the 5th, 6th, or 7th week, a little later than the epiphyses of the other ossa metacarpalia (Table 2).

The os metatarsale 1 varies considerably in the degree to which it is developed. In all of the dogs studied, it was small and developed from a single center of ossification, which appeared during the 6th, 7th, 8th, or 9th week (Table 2).

Ossa Digitorum Manus et Pedis.—The phalanges proximales and phalanges mediae each develop from 2 centers of ossification, one for the corpus et caput phalangis (diaphysis) and one for the basis phalangis (epiphysis proximalis).

The centers for the diaphyses are present at birth. The centers for the epiphyses of the phalanges proximales II–V appear during the 4th, 5th, 6th, or 7th week, and those of the phalanges mediae II–V appear some time from the 5th to the 9th week. The epiphyses in the pectoral limb appear before those in the pelvic limb (Fig. 3, 4), and the epiphyses of the axial digits before those of the abaxial digits. The phalanx proximalis I also develops from 2 centers of ossification. The center for the epiphysis proximalis appears during the 5th, 6th, 7th, or 8th week, or about the same time that the epiphyses of the phalanges mediae appear.

The phalanx distalis of each digit develops from a single center of ossification, which is present at birth.

Ossa Sesamoidea.—The ossa sesamoidea in the pectoral limb are the phacoid, a pair of ossa sesamoidea volaria at each articulatio metacarpophalangae except that of the 1st digit where there is only one os sesamoideum, and an os sesamoideum dorsale at each articulatio metacarpophalangae. The ossa sesamoidea in the pelvic limb are the patella, the fabellae, the os sesamoideum popliteum, a pair of ossa sesamoidea plantaria at each articulatio metatarsophalangeae except that of the 1st digit, unless it is fully developed, and an os sesmoideum dorsale at each articulatio metatarsophalangeae.

Each os sesamoideum develops from a single center of ossification. The center for the patella appears during the 2nd, or early in the 3rd, month. The centers for the ossa sesamoidea volaria and the ossa sesamoidea plantaria appear sometime from the 7th to the 15th week. The centers for the fabellae appear during the 3rd, or early in the 4th, month, and those for the phacoid, the os sesamoideum popliteum, and the ossa sesamoidea dorsalis during the 4th or the 5th month (Table 2).

Discussion

The time of the appearance of the ossification centers that appear after birth has been studied roentgenographically, because this method has the obvious advantage that the ossification process can be followed throughout the growth stages of an individual animal. Although the number of dogs used was limited, it has been possible to establish when the centers appear more accurately than had been previously determined. This is particularly true for those bones for which the previous available information was either very general or inconclusive: namely, the bones of the limbs proximal to the manus and pes.

One fact which has emerged is that the age at which any center appears is variable. In the breeds studied, the variation is quite marked among animals from different breeds, and it is also evident to a lesser degree among animals from the same breed or litter. For example, the ossification centers generally appear at an earlier age in the German Shepherd Dog and the Collie than they do in the Beagle or the Bulldog (Table 2). There are also obvious individual variations in the Collie, which were seen when the dogs were examined at short intervals during the 1st month of life. Pomriaskinsky-Kobozieff and Kobozieff [13] and Bressou *et al.*[2] noticed similar breed and individual variations, but of a lesser degree, during their study of the manus and pes. The difference in the time of appearance of an ossification center in animals from different breeds, or from the same breed or litter, is usually less for the centers that appear early in life than it is for those that appear later in life. In Table 2, this difference may have been exaggerated for the centers that appear later in life, because the interval between the examinations was increased as the animals grew older; therefore, the figures were not so accurate. However, even after allowance has been made for this, it is clear that there is an increase in the difference as the animals grow older. Some variation in the rate of ossification between normal dogs is understandable and to be expected. In man, it is well known that there are racial and familial differences in the rate of ossification, and a recent study by Breitbach [1] has provided further evidence that the beginning of ossification is dependent on hereditary influence. Also, in the sheep, Smith [21] has observed that, in twin and triplet fetuses, ossification is more advanced in the larger fetuses.

Although there are variations in the ages at which the ossification centers appear, the chronologic order of the appearance of the centers remains fairly constant, granting that there are exceptions, particularly among the bones of the carpus and tarsus and, in the Bulldog, between the epiphyses of the ossa metatarsalia and the phalanges proximales (Table 2).

Sex does not seem to influence the ossification process in the dog as it does in the primates, in the rat, and, perhaps, in the sheep. The findings of this study have shown that the ossification process is sometimes more advanced in the male, but that the reverse may also be found. However, the differences, when they occur, are slight, and the evidence is inconclusive, because a large-scale investigation would be required to determine the influence of sex on the rate of ossification. Pomriaskinsky-Kobozieff and Kobozieff [13] and Bressou *et al.*[2] were inclined to the view that the ossification process was more advanced in the male, but they admitted that their evidence was inconclusive because of the small number of animals studied. If it should subsequently be shown that, in the dog, the rate of ossification is more advanced in the male than in the female, it will open up an interesting field of study, because, in the primates and the rat, it has been observed that the ossification process is more advanced in the female. Among others, Pryor,[14, 16] Menees and Holly,[11] Francis *et al.*, [6] Hill,[9] and Pyle and Sontag [17] have reported that the ossification process in man is more advanced in the female than in the male. Similar observations have been made for the chimpanzee by Nissen and Riesen, [12] for the rhesus monkey by van Wagenen and Asling,[24] and for the rat by Spark and Dawson.[22] In the sheep, Smith [21] observed that, if twin and triplet fetuses were of the same size, the ossification process was more advanced in the female. However, as mentioned earlier, if the fetuses were of different sizes, the ossification process was more advanced in the larger fetus.

In several epiphyses, the ossification process has been seen to start simultaneously at 2 points. The epiphyses in which this has been observed are the epiphysis proximalis ulnae, the epiphysis distalis ulnae, the epiphysis distalis fibulae, and the tuber calcanei. Schaeffer,[19] Pomriaskinsky-Kobozieff and Kobozieff,[13] and Bressou *et al.*[2] have also noticed that ossification sometimes starts simultaneously at more than one point. Francis *et al.*[6] have reported a similar occurrence in man and have attributed it to an asymptomatic form of osteochondrosis. When ossification starts at more than one point, the points are normally situated close together and usually unite shortly after they appear. If, however, the points were to fail to fuse at an early date, it would appear as though there were 2 centers of ossification. This may account for the fact that the author, like Schaeffer,[19] has been able to demonstrate only one center of ossification for the epiphysis proximalis humeri, either roentgenographi-

cally or by staining and then clearing the bone, whereas Lesbre,[10] Ellenberger and Baum,[4] and Seoudi [20] consider that there is a separate center for the tuberculum majus. Failure of separate points of ossification to unite and form a single center of ossification may also account for such clinical entities as a bipartite patella, or an accessory center at the head of the radius such as that noticed by Cawley and Archibald.[3]

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SUMMARIO IN INTERLINGUA

Le Etates al Quales le Centros de Ossification Appare in le Ossos del Extremitates in le Can

Periodos de etate al quales centros de ossification appare post nato in le ossos del extremitates del can esseva determinate per roentgenographia in subjectos de quatro racias, i.e. canes de pastor german, colleys, bull-dogs, e bigles. Vinti-quatro canes esseva examinate durante le prime 3 septimanas de lor vitas, e 14 de istos esseva observate additionalmente durante le sequente 5 septimanas de lor vitas.

Le centros de ossification appare in approximativemente le mesme ordine chronologic, sed le etates al quales illos appare es subjecte a variationes. Tal variationes pote esser presente in animales del mesme portata e del mesme racia. Illos occurre plus marcatemente in individuos de differente racias. Le sexo del animales non pare influentiar le etate al qual le centros appare.

The Antigenic Structure of Virulent and Avirulent Strains of *Erysipelothrix rhusiopathiae*. I. Immunobiologic Properties

Marian Truszczynski, Dr. Med. Vet.

SUMMARY

Immunogenic differences were demonstrated among strains A, B, StG, and StF of *Erysipelothrix rhusiopathiae*. Formalin-inactivated strain B was moderately immunogenic for mice, whereas formalin-killed strains A, StG, and StF did not immunize mice against challenge with 5 to 10 LD_{50} of strain R203.

In contrast to the results obtained with formalin-killed *Ery. rhusiopathiae*, 111 live organisms of strain StF were capable of protecting mice against infection with virulent strain R203. However, 61.8 million live organisms of strain StG did not protect mice against a similar challenge dose of strain R203. Similar results were obtained in pigs with the same 2 avirulent strains of *Ery. rhusiopathiae*. Strain StF adequately immunized pigs against challenge by the method of Fortner-Dinter for as long as 4½ months after vaccination. Strain StG failed to immunize pigs under similar conditions.

INTRODUCTION

The purpose of the work reported here was to test the immunogenic properties, for mice, of 4 formalin-killed strains of *Erysipelothrix rhusiopathiae*, and to determine the immunogenic properties, for mice and pigs, of 2 live avirulent strains of *Ery. rhusiopathiae*. This work is an introduction to further experiments concerning the immunochemic and serologic properties of these strains.[6]

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Materials and Methods

Four strains of *Ery. rhusiopathiae* were used. Strains A and B were virulent and belonged to A and B serotypes, respectively, according to Dedie.[1,2] They were isolated from swine in the Veterinary Institute in Pulawy, Poland. Strains StG and StF, both belonging to serotype B, were avirulent strains derived from Staub's strains.[4,5] The 4 strains of *Ery. rhusiopathiae*, A, B, StG, and StF, were each cultured for 48 hours in horse-meat broth containing 10% horse serum. Each culture was killed with formalin at a final concentration of 0.4% and concentrated by centrifugation to ⅛ of the original volume. Five tenths of a milliliter of each of the 4 cultures was given subcutaneously to each of 20 mice.* Fourteen days later, the immunity of the mice was challenged by subcutaneous injection of an 18-hour broth culture of a virulent strain of *Ery. rhusiopathiae* (strain R203, serotype A,5). The challenge dose for each mouse contained 4 to 8 organisms from this broth culture diluted in 0.85% NaCl solution (5 to 10 LD_m).

TABLE 1—Mouse Protection Tests with Live Erysipelothrix rhusiopathiae Strains StF and StG

Dilution of broth culture for vaccination of mice	No. of microorganisms/ml.*		Challenge**	
	Strain StF	Strain StG	Strain StF	Strain StG
10^{-2}	.....	.....	6/6	0/8
10^{-3}	.....	.....	8/8	0/8
10^{-4}	.....	.....	8/8	0/8
10^{-5}	.....	.....	8/8	0/8
10^{-6}	444.6	247.2	8/8	0/8
10^{-7}	36.0	20.6	0/8	0/8

* These are average numbers obtained by counting microorganisms in triplicate plates from dilutions 10^{-6} and 10^{-7}.

** The immunity of all mice was challenged 14 days after vaccination with 0.2 ml. of virulent strain R203 cultured for 24 hours. Numerator indicates number of mice surviving; denominator indicates number whose immunity was challenged.

Living organisms of strains StG and StF, used for vaccination of mice, were cultivated for 24 hours in horse-meat broth containing 3% horse serum. After incubation, cultures were diluted in tenfold serial dilutions. Each dilution of 10^{-2} through 10^{-7} was injected in 0.25 ml. amounts into

* Swiss strain mice bred at the Veterinary Institute, Pulawy.

8 mice. From dilutions 10^{-6} and 10^{-7}, viable organisms were counted by the plate method to determine the number inoculated. Fourteen days later, the mice were inoculated with 0.2 ml. of the virulent R203 strain in the dose of 1:20 dilution of the 24-hour culture.

Five healthy pigs of the Pulawy breed, weighing 50 to 70 kg., were vaccinated subcutaneously with 2.0 ml. of strain StG, grown for 24 hours in horse-meat broth with 3% horse serum. Ten pigs of the same breed and weight were vaccinated subcutaneously with strain StF grown in the same way. Three weeks later, immunity of the pigs was challenged with strain R203, using the method of Fortner-Dinter.[3] The local skin reactions and the general reactions of the infected pigs were evaluated as described in a previous report.[5]

Results

All mice vaccinated with formalin-killed strains A, StG, and StF died after challenge with strain R203. Strain B was more active immunologically because 25% of the vaccinated mice were protected against 5 to 10 LD_{50} of strain R203.

The results of the experiments with mice, using the live, avirulent strains, are shown in Table 1. All of 20 mice (not shown in Table 1) vaccinated with undiluted culture of strain StG died following challenge with virulent *Ery. rhusiopathiae*, indicating that even 61.8 million organisms were not sufficient to immunize mice against infection with 0.2 ml. of the 24-hour culture of virulent strain R203, diluted 1:20. In the case of strain StF, it was found that 111 organisms were sufficient to immunize mice against the same challenge dose of R203.

TABLE 2—Reaction of Pigs to Challenge by the Method of Fortner and Dinter 3 Weeks After Vaccination with Live Erysipelothrix rhusiopathiae Strains StG and StF

	Postchallenge day 1		Postchallenge day 2		Postchallenge day 3	
Pig	Skin reaction	Body temperature (C.)	Skin reaction	Body temperature (C.)	Skin reaction	Body temperature (C.)
1	+	38.6	++	41.6	++++	42.0
2	—	40.7	++++	40.8	++++	41.8
3	+	40.8	+	41.7	+++	40.8
4	—	39.7	+++	40.7	+++	41.8
5	—	40.2	+	42.0	+	41.5
6	—	39.0	—	39.3	—	39.0
7	—	38.7	—	40.0	—	39.2
8	—	38.9	—	39.0	—	38.7
9	—	38.9	—	38.7	—	38.3
10	—	39.4	—	39.0	—	39.1
11	—	39.1	—	38.4	—	38.5
12	—	38.3	—	38.8	—	38.8
13	—	38.6	—	38.8	—	38.6
14	—	38.7	—	38.9	—	39.0
15	—	39.0	—	39.0	—	38.7

Pigs 1 through 5 were vaccinated with strain StG; pigs 6 through 15 were vaccinated with strain StF.

The results of the vaccination of pigs with strain StG and StF are shown in Table 2. Five vaccinated pigs, used as controls for determining the virulence of the R203 strain, developed typical signs of erysipelas. Strain StG failed to protect pigs against challenge with virulent *Ery. rhusiopathiae;* on the other hand, strain StF was found to give good protection against infection by the method of Fortner-Dinter for as long as 4½ months after vaccination.[5]

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SUMMARIO IN INTERLINGUA

Le Structura Antigenic de Virulente e Avirulente Racias de *Erysipelothrix rhusiopathiae*. I. Proprietates Immunobiologic

Differentias immunogenic esseva demonstrate inter le racias A, B, StG, e StF de *Erysipelothrix rhusiopathiae*. Racia B, inactivate per formalina, esseva moderatemente immunogenic pro muses, durante que racias A, StG, e StF, similemente inactivate, non immunisava muses contra un provocation con racia R203 in doses de 5 a 10 DL_{50}.

Per contrasto con le resultatos obtenite con *Ery. rhusiopathiae* post inactivation con formalina, 111 organismos vive del racia StF sufficeva a proteger muses contra le infection con virulente racia R203. Tamen, 61,8 milliones organismos vive del racia StG non esseva capace a proteger muses contra un simile dose provocatori de racia R203. Simile resultatos esseva obtenite in porcos con le mesme 2 avirulente racias de *Ery. rhusiopathiae*. Racia StF immunisava adequatemente porcos contra le provocation per le methodo de Fortner-Dinter durante periodos de usque a 4½ menses post le vaccination. Racia StG non immunisava le porcos sub simile conditiones.

The Antigenic Structure of Virulent and Avirulent Strains of *Erysipelothrix rhusiopathiae*. II. Immunochemic and Serologic Investigations

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SUMMARY

Chemical fractionation of *Erysipelothrix rhusiopathiae* organisms from 4 strains differing in type-specific haptens, immunogenic properties, and virulence, resulted in the isolation of 3 polysaccharide fractions (C_1, C_{1T}, and C_2) and one nucleoprotein fraction (Np_1) for each strain. Strains B and StF, which had marked immunogenic ability, yielded 2 additional fractions (Np_2 and Np_3).

Chromatographic analysis revealed glucosamine, xylose, and galactose in fractions C_1, and galactose only in fractions C_2. No differences were found by chromatography between polysaccharides derived from different strains.

None of the fractions were immunogenic or toxic when given subcutaneously to mice. The bacterial fractions did not absorb protective antibodies from a horse anti-erysipelas serum. All of the bacterial fractions were serologically active in the hemagglutination test. Fraction C_2, however, was active with anti-erysipelas serum of swine origin only, whereas the other fractions were active with horse and rabbit serum also. It was found that fractions C_1 and C_{1T} contained 1 type-specific and 2 species-specific components varied with the bacterial strain. The bacterial fractions fixed complement in the presence of anti-erysipelas serum from rabbits and swine, but not from horses.

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INTRODUCTION

In a preceding report,[14] the immunobiologic properties of strains A, B, StG, and StF of *Erysipelothrix rhusiopathiae* were described. The present report is concerned with the chemical fractionation of the same strains of *Ery. rhusiopathiae* and with the chromatographic, immunobiologic, and serologic properties of the fractions obtained.

Materials and Methods

The methods used for fractionation of strains A, B, StG, and StF were described previously.[8] Briefly, the method was as follows: A heavy bacterial suspension of strains A, B, StG, and StF of *Ery. rhusiopathiae* in Koch's solution (0.85% NaCl and 0.5% phenol, in water) was frozen and thawed about 10 times to cause lysis of the bacteria. The pH of the suspension was adjusted to 4.5 by addition of acetic acid, followed by extraction in the refrigerator overnight. Then the supernatant fluid was separated from the bacteria by centrifugation. The active material was precipitated from the supernatant fluid by the addition of 2 volumes of ethanol; after purification and drying, this was called the C_1 fraction (depending on the strain: C_1A, C_1B, C_1G, C_1F). The sediment (x) was mixed with 0.1 N NaOH and, after some hours, the supernatant fluid and sediment were again separated by centrifugation. The supernatant fluid contained mainly proteins. Glacial acetic acid was added until precipitation occurred (about pH 4). The precipitate, separated by centrifugation, was found to be a nucleoprotein[3] and was labeled the Np_1 fraction. The remaining supernatant fluid was treated with trichloracetic acid, and a precipitate called Np_2 was obtained; a further addition of trichloracetic acid gave the next precipitate, Np_3. These fractions (Np_2 and Np_3), because of their precipitation with trichloracetic acid, were assumed to be mainly protein in nature. The sediment marked x (above) was mixed with 10% NaOH solution and boiled 30 minutes; it was filtered, precipitated by ethanol (2 volumes), redissolved in distilled water, precipitated again with ethanol, and dried. This was called the C_2 fraction. For chromatographic investigations, the method of Dzulynska and Mikulaszek[5] was used.

The immunobiologic properties of the bacterial fractions were tested in every case by inoculation

of 2 mice with 5 mg. of material. Only 2 mice were used because of the small quantity of material available. The polysaccharide fractions were diluted in physiologic NaCl solution, and the protein fractions in 0.1 N NaOH, followed by addition of enough 0.1 N HCl to reach pH 7. Fourteen days later, the immunity of the mice was challenged by injecting 5 to 10 LD_{50} of virulent strain R203 of *Ery. rhusiopathiae*.[14] The organs of the mice that died were cultured on blood-agar plates.

A technique was devised to test the ability of the bacterial fractions to absorb from an immune serum the antibodies responsible for the protection of mice infected with strain R203 of *Ery. rhusiopathiae*. One milliliter of a polyvalent horse anti-erysipelas serum was mixed with 1 milliliter of a solution containing 10 mg. of the bacterial fraction to be tested. The mixture was incubated for one hour at 37 C., then overnight at 4 C. The mixture was then centrifuged and the clear supernatant fluid diluted in twofold steps from 1:12.5 to 1:800.0. Into 5 mice were injected 0.25 ml. of each dilution of absorbed serum and 5 to 10 LD_{50} of strain R203. The results were evaluated as described by Kaplan.[7] Serologic studies were conducted on the bacterial fractions by the hemagglutination test, according to a method described previously.[13] The following antiserums were used: polyvalent (anti-A and anti-B) anti-erysipelas horse serum, precipitating anti-A and anti-B serums of swine origin, and anti-A and anti-B precipitating serums of rabbit origin. The sheep red blood cells were sensitized with 2.0, 1.0, 0.2, or 0.1 mg. of bacterial fraction per milliliter of 10% red blood cell suspension. The activity of the fractions was tested routinely against a standard anti-erysipelas serum.

The complement-fixation test was performed according to the technique of Osler *et al.*[9] Anti-A and anti-B rabbit serums, the anti-B swine serum, and the horse polyvalent (anti-A and anti-B) serum were used. Constant doses of immune serum and twofold dilutions of antigen, beginning with a dilution of 1:1,000, were used. Before addition of sensitized cells, the tubes were incubated for 30 minutes in a water bath at 37 C. The hemolytic system was then added, and the tubes were reincubated at 37 C. The test was read after complete hemolysis in the antigen and serum controls. The dilution of antigen at which there was complete inhibition of red blood cell lysis was regarded as the end point.

Results

The fractions isolated from the various strains of *Ery. rhusiopathiae* are listed in Table 1. Some differences were found among the individual strains. No Np_2 and Np_3 fractions were found in strains A and StG. Because of the relatively small quantities of the fractions, chemical analyses were not performed. However, analyses of fractions C_1, C_2, and Np_1 of serotype A were reported previously.[3] The total amount of nitrogen, phosphorus, desoxyribose (as DNA with diphenylamine), and ribose (as RNA with orcinol), as well as the over-all amount of carbohydrates (Dische's *alpha*-naphthol method), protein (Machoboeuf's xanthroproteic method), and hexosamines (Morgan-Elson method), was determined. The chemical structure of the serologically active antigens was also characterized by quantitative precipitation[3] and determination of polysaccharides and nucleoproteins in the washed precipitates. On the basis of these results, as well as precipitability in ethanol, acetic acid, and trichloracetic acid, and the co-precipitation test with Wassermann's antigen, the C_1 fraction was found to be polysaccharide combined with protein and lipids. Fraction C_2 was found to be polysaccharide. Fraction Np_1 was an isoelectric nucleoprotein. Since the same methods of fractionation were used, it was believed that fractions C_1, C_{1T}*, C_2, and Np_1, obtained in this work, had the same structure as fractions C_1, C_2, and Np_1, described previously.[3] Since the Np_2 and Np_3 fractions were obtained by precipitation with trichloracetic acid, it may be assumed that they were proteins or had a protein component.

TABLE 1—Fractions Obtained from 4 Strains of Erysipelothrix rhusiopathiae

Strain	Symbols of the fractions obtained					
A	C_1	C_{1T}	Np_1	.....	.....	C_2
B	C_1	C_{1T}	Np_1	Np_2	Np_3	C_2
StG	C_1	C_{1T}	Np_1	.....	.....	C_2
StF	C_1	C_{1T}	Np_1	Np_2	Np_3	C_2

Chromatographically, it was found that fractions C_1 and C_{1T}, irrespective of the strains from which they originated, contained the same monosaccharides; namely, galactose, xylose, and glucosamine. All of the C_2 fractions contained only galactose, regardless of the strains from which they originated, and had a galactanic structure.

Immunobiologic studies were conducted on fractions of the A, B, StG, and StF strains. It was found that none of these fractions injected into mice in doses of 5 mg. protected them against subsequent inoculation with strain R203.

* The chemical preparation of fractions C_1 and C_{1T} differed at one point only: i.e., for the C_{1T} fractions, the acid extracts of bacteria obtained with acetic acid at pH 4.5 were concentrated by boiling before precipitation with ethanol.

Experiments on the absorption of protective antibodies from the anti-erysipelas horse serum by the individual fractions were carried out on 806 mice. In order to better interpret the results, the 50% effective dose (ED_{50}) of the nonabsorbed and absorbed serums was calculated according to Kaplan.[7] The results are summarized in Table 2.

TABLE 2—Dose of Anti-Erysipelas Horse Serum Which Protected 50% of the Mice Exposed to 5 to 10 50% Lethal Doses of Erysipelothrix rhusiopathiae (ED_{50})

Serum absorbed with fraction	ED_{50} of absorbed serum (ml.)	ED_{50} of nonabsorbed serum (ml.)
C_1A	0.00167	0.000931
Np_1A	0.00222	
C_1B	0.00167	0.000881
Np_1B	0.00222	
C_1G	0.00280	0.00167
Np_1G	0.00280	
C_1F	0.00164	0.00157
Np_1F	0.00201	
Np_2B	0.00555	0.00176
Np_3B	0.00186	
Np_2F	0.00705	0.00111
Np_3F	0.00155	
C_2A	0.000769	
C_2B	0.00267	0.00111
C_2G	0.00155	
C_2F	0.00130	

The protective properties of the immune serum absorbed with fractions Np_1A, Np_1B, C_1G, Np_1G, Np_2B, Np_2F, and C_2B were lower than those of the unabsorbed serum. The differences were not too distinct, possibly due to error of method. It was therefore concluded that the fractions examined did not absorb from anti-erysipelas serum the antibodies responsible for protection of mice against inoculation with 5 to 10 LD_{50} of strain R203.

The results of the hemagglutination test of the polysaccharide and protein fractions against polyvalent horse, swine (type-specific anti-A and anti-B), and rabbit anti-A and anti-B serums are summarized in Table 3.

The serologic activities in the hemagglutination test of the C_1 and C_{1T} fractions, tested against polyvalent anti-erysipelas horse serum and the anti-A and anti-B swine or rabbit serums, were generally similar. The titers of the serums ranged from 1:1024 to 1:4096. The Np_1 fractions tested against the same serums were less active, and fractions Np_2B, Np_3F, Np_3B, and Np_2F were least active. The C_2 fractions were not serologically active against anti-erysipelas horse and rabbit serums, but they were active against the anti-A and anti-B swine serums. The titers obtained ranged from 1:8 to 1:16. Experiments carried out with type-specific swine serums anti-A and anti-B, and the C_1 fractions as antigens, revealed no differences in titers, regardless of whether the homologous or heterologous serotype was being tested. The results indicated that these fractions contained, besides type-specific antigens,[3] a factor common to both serotypes. The latter obscured the type-specificity of the antigens.

TABLE 3—Hemagglutination Titers of Horse, Swine, and Rabbit Anti-Erysipelas Serums Tested with Various Bacterial Fractions

Fraction	Type and titers of serum				
	Horse anti-A + B	Swine anti-A	Swine anti-B	Rabbit anti-A	Rabbit anti-B
C_1A	1:2,048	1:1,024	1:2,048	1:32	1:4,096
C_1B	1:2,048	1:512	1:1,024	1:32	1:2,048
C_1G	1:2,048	1:1,024	1:1,024	1:32	1:1,024
C_1F	1:256–1:1,024	1:64	1:64	—	1:128
C_1A_T	1:2,048	1:2,048	1:2,048	1:64	1:2,048
C_1B_T	1:2,048	1:128	1:512	1:32	1:2,048
C_1G_T	1:2,048	1:1,024	1:2,048	1:32	1:2,048
C_1F_T	1:2,048	1:1,024	1:1,024	1:32	1:2,048
Np_1A	1:512	1:512	1:512	1:16	1:1,024
Np_1B	1:1,024	1:1,024	1:1,024	1:32	1:2,048
Np_1G	1:512	1:512	1:512	1:16	1:512
Np_1F	1:512	1:128	1:128	1:16	1:512
C_2A	—	1:8	1:8	—	—
C_2B	—	1:8	1:8	—	—
C_2G	—	1:8	1:8	—	—
C_2F	—	1:16	1:8	—	—
Np_2B	1:256	1:8	1:8	—	1:32
Np_3B	1:64	1:32	1:8	—	1:16
Np_2F	1:64	1:8	1:8	—	1:16
Np_3F	1:256	1:8	1:8	—	1:16

It was impossible to detect the type-specific antigen by increasing the concentration of the sensitizing antigenic fractions from 1:10,000 to 1:500. Similar titers were obtained with the homologous and heterologous systems.

In another experiment, anti-erysipelas horse serum was absorbed by each individual fraction, and the hemagglutination test was carried out using all of the fractions. The results are summarized in Table 4. This experiment showed that all except the C_2 fractions contained 2 common antigens which were able to cause hemagglutination. They were designated as X and Y components. The C_1 and C_{1T} fractions of the A, B, and StF strains contained these components in the same proportion, while the C_1 and C_{1T} fractions of the StG strain (C_1G and C_1G_T) contained them in a different proportion. The above mentioned 2 fractions (C_1G and C_1G_T) probably had more Y component than the C_1 and C_{1T} fractions of the A, B, and StF strains.

The results of the complement-fixation

TABLE 4—Hemagglutination Titers of Anti-Erysipelas Horse Serum Absorbed and Not Absorbed by Individual Fractions and Tested with Fractions of 4 Strains of Erysipelothrix rhusiopathiae

Absorbing fractions*	Erythrocytes sensitized with fraction										
	C_1A	C_1B	C_1G	C_1F	C_1A_T	C_1B_T	C_1G_T	C_1F_T	C_2A	C_2B	C_2G
Serum not absorbed	2,048	2,048	2,048	128–1,024	2,048	2,048	2,048	2,048	—	—	—
Serum absorbed by:											
C_1A	—**	—	—	—	—	—	32	—	—	—	—
C_1B	—	—	—	—	—	—	32	—	—	—	—
C_1G	256	256	—	64	512	64	—	256	—	—	—
C_1F	128	64	128	—	1,024	64	256	64	—	—	—
C_1A_T	—	—	—	—	—	—	—	—	—	—	—
C_1B_T	—	—	64	—	—	—	64	—	—	—	—
C_1G_T	512	256	—	32	512	256	—	256	—	—	—
C_1F_T	—	—	128	—	—	—	64	—	—	—	—
C_2A	1,024	2,048	1,024	512	2,048	1,024	1,024	2,048	—	—	—
C_2B	2,048	2,048	2,048	1,024	2,048	2,048	2,048	2,048	—	—	—
C_2G	1,024	1,024	512	2,048	2,048	2,048	2,048	2,048	—	—	—
C_2F	2,048	2,048	1,024	64	2,048	2,048	2,048	2,048	—	—	—
Np_1A	512	128	128	—	512	256	512	512	—	—	—
Np_1B	128	—	—	—	128	64	256	64	—	—	—
Np_1G	1,024	128	—	—	1,024	512	128	1,024	—	—	—
Np_1F	1,024	—	512	—	1,024	64	256	128	—	—	—
Np_2B	512	128	256	—	1,024	256	512	128	—	—	—
Np_2B	1,024	1,024	1,024	1,024	2,048	1,024	1,024	1,024	—	—	—
Np_2F	1,024	1,024	1,024	512	1,024	1,024	1,024	1,024	—	—	—
Np_2F	1,024	256	256	—	512	512	2,048	512	—	—	—

Absorbing fraction*	Erythrocytes sensitized with fraction								
	C_2F	Np_1A	Np_1B	Np_1G	Np_1F	Np_2B	Np_2B	Np_2F	Np_2F
Serum not absorbed	—	512	1,024	512	512	256	64	64	256
Serum absorbed by:									
C_1A	—**	—	—	—	—	—	—	—	—
C_1B	—	—	—	—	—	—	—	—	—
C_1G	—	—	128	—	—	—	—	—	—
C_1F	—	—	—	—	—	—	—	—	—
C_1A_T	—	—	—	—	—	—	—	—	—
C_1B_T	—	—	—	—	—	—	—	—	—
C_1G_T	—	—	128	—	—	—	—	—	—
C_1F_T	—	—	—	—	—	—	—	—	—
C_2A	—	256	256	512	256	—	—	—	—
C_2B	—	256	1,024	1,024	1,024	—	16	—	—
C_2G	—	1,024	1,024	1,024	1,024	—	16	—	—
C_2F	—	256	1,024	128	256	—	16	—	—
Np_1A	—	—	—	—	—	—	—	—	—
Np_1B	—	—	—	—	—	—	—	—	—
Np_1G	—	—	128	—	—	—	—	—	—
Np_1F	—	—	—	—	—	—	—	—	—
Np_2B	—	—	32	—	—	—	—	—	—
Np_2B	—	256	512	256	256	—	—	—	—
Np_2F	—	256	512	256	256	—	—	—	—
Np_2F	—	—	256	—	—	—	—	—	—

* The absorption technique used was the same as that for experiments listed in Table 2.
** Indicates no hemagglutination in a dilution of 1:16.

test with the fractions of the A, B, StG, and StF strains, and anti-erysipelas serums of rabbit, pig, and horse, are summarized in Table 5.

type-specificity, they did not differ in the simple saccharides present. This was not unexpected, since serologic specificity depends not only on the composition of simple

TABLE 5—Complement-Fixation Titers of Fractions of Strains A, B, StG, and StF Against Anti-Erysipelas Serum of Horse, Pig, and Rabbit Origin

Fraction	Horse serum anti-A + B	Swine serum anti-B	Rabbit serum anti-A	Rabbit serum anti-B
C_1A	—	—	1:16,000	1:512,000
C_1B	—	1:8,000	1:16,000	1:256,000
C_1G	—	1:8,000	1:16,000	1:256,000
C_1F	—	—	1:4,000	1:64,000
C_1A_T	—	—	1:32,000	1:256,000
C_1B_T	—	1:8,000	1:16,000	1:256,000
C_1G_T	—	1:8,000	1:16,000	1:128,000
C_1F_T	—	1:8,000	1:16,000	1:128,000
Np_1A	—	1:16,000 KA —	1:8,000 KA —	1:32,000 KA —
Np_1B	—	1:64,000 KA 1:8,000	1:32,000 KA 1:8,000	1:256,000 KA 1:8,000
Np_1G	—	1:32,000 KA 1:1,400	1:16,000 KA 1:4,000	1:128,000 KA 1:4,000
Np_1F	—	1:32,000 KA 1:2,000	1:8,000 KA 1:2,000	1:64,000 KA 1:2,000
C_2A	—	.....	—	1:8,000
C_2B	—	.....	1:16,000	1:256,000
C_2G	—	.....	—	—
C_2F	—	.....	1:16,000	1:128,000

KA = antigen control.

All C_1 and C_{1T} fractions, except the C_1F fraction, had the greatest activity against anti-erysipelas rabbit serums (anti-A and anti-B), followed by the Np_1B and C_2B fractions, and finally by the remaining fractions Np_1 and C_2. No differences in the titers of the type-specific rabbit serums were found with the individual C_1 fractions. The Np_1 fractions had anticomplementary activity. The C_1 and C_{1T} fractions, tested against anti-B swine serum, were more active than the Np_1 fractions. The C_2 fractions were not tested. None of the fractions tested with the complement-fixation test against anti-erysipelas horse serum were serologically active.

Discussion

The discovery that strains B and StF possess 2 fractions (Np_2 and Np_3) which are absent in strains A and StG may be significant, since strains B and StF are also more effective in immunizing mice and swine.[14] It is possible that fractions Np_2 and Np_3 contain the antigens responsible for protective immunity. Similar investigations on a larger number of strains of *Ery. rhusiopathiae* should make it possible to correlate chemical structure with immunogenic properties.

The results obtained with paper chromatography demonstrated that, although the C_1 fractions contained antigens of different saccharides, but also on many other chemical properties.[16] Although the C_2 fractions also differed in their antigenic composition,[17] they did not differ chromatographically. The investigations confirmed the previous results,[3] in which the 2 chemically different polysaccharide haptens of serotype A contained in fractions C_1 and C_2, were examined.

The finding that none of the fractions obtained, including fractions Np_2 and Np_3, had immunizing properties, does not weaken the argument for the role of fractions Np_2 and Np_3 in immunity. It is possible that the chemical manipulations, especially precipitation with trichloracetic acid, destroyed the biologic activity of these fractions. Special methods of preparation, similar to those described for the anthrax bacillus [1,2,12] should be employed, as well as strains characterized by good immunogenic properties, even when used as formalin-killed bacterins.

The results of the hemagglutination test obtained with the C_1 fractions and type-specific serums should be discussed in comparison with those reported in a previous paper.[3] In the latter work, the C_1 fraction of serotype A was tested by means of the quantitative precipitation test, and it was demonstrated that the polysaccharide hapten C_1 was responsible for type-specificity. This could not be confirmed with the hemagglutination test, since similar titers

were obtained with fractions C_1 of serotypes A and B with the various type-specific serums. It was shown later by means of the gel-diffusion test [15] that both type-specific and species-specific antigens were present in fraction C_1. In the hemagglutination test, the species-specific component was probably more active than the type-specific one, and masked it.

It was interesting that the C_2 fractions, although active with horse- and rabbit-immune serum in the precipitation test, were inactive in the hemagglutination test with the same serums. On the other hand, they produced positive reactions in the hemagglutination test (titers 1:8 to 1:32) with anti-erysipelas swine serum. These results indicate that there was no lack of receptors for the antigenic substances of the C_2 fractions on the surface of the erythrocytes, but that there were differences in the level of anti-C_2 antibodies between the rabbit and horse serums on one hand, and the swine serum on the other. The 3 previously mentioned serums had similar titers when tested with erythrocytes sensitized with C_1 fractions. Experiments carried out with type-specific swine serums, and the C_2 fractions as antigens, showed that there were no differences in titers whether or not the sensitizing fraction was derived from the homologous or the heterologous serotype. This indicates that the C_2 fractions also contain a common component active in the hemagglutination test, besides antigenic differences detected with the gel-precipitation test.[15]

The hemagglutination test, with anti-erysipelas horse serum absorbed with various bacterial fractions, disclosed differences in the amounts of the X and Y components of the species-specific antigen (fractions C_1 and C_{1T}) from the StG strain, as compared to the amounts from the other strains. This finding may be significant in that the StG strain was the least immunogenic of the strains used.

Absorption of the horse anti-erysipelas serum with fractions C_1, Np_1, Np_2, and Np_3, removed both precipitating and hemagglutinating antibodies to these fractions, indicating that the same antibody was detected with the 2 techniques.

The results obtained with the complement-fixation test were generally similar to those of the hemagglutination test. However, the anti-erysipelas horse serum which possessed high hemagglutination and precipitation titers was inactive in the complement-fixation test with all the fractions. Schaeffer and Sandor [10] demonstrated precipitation and fixation of complement with rabbit-immune serums and capsular and somatic antigens of the anthrax bacillus, but only precipitation with horse-immune serum. Analogous observations were made with polysaccharides from pneumococci. Mikulaszek and Ratomski [8] demonstrated that the whole bacteria and one of the polysaccharide fractions of *Salmonella abortus equi* caused strong complement-fixation with horse serums containing significant amounts of somatic precipitins and agglutinins, whereas the remaining polysaccharide fractions obtained by various methods were inactive in the CF test with horse anti-*Salmonella* serum. These authors assumed that the antibody in horse serum was active only when tested with substances possessing the characteristics of a complete antigen or endotoxin, or with the whole bacteria.

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SUMMARIO IN INTERLINGUA

Le Structura Antigenic de Virulente e Avirulente Racias de *Erysipelothrix rhusiopathiae*. II. Investigationes Immunochimic e Serologic

Le fractionation chimic de organismos de *Erysipelothrix rhusiopathiae* ab 4 racias con differentias in haptenos a specificitate per typo, in proprietates immunogenic, e in virulentia resultava in le isolation de 3 fractiones polysaccharidic (C_1 C_{1T}, e C_2) e de un fraction nucleoproteinic (Np_1) pro cata un del racias. Racias B e StF, que habeva marcate capacitate immunogenic, rendeva 2 fractiones additional (Np_2 e Np_3).

Le analyse chromatographic revelava glucosamina, xylosa, e galactosa in fraction C_1 e galactosa sol in fraction C_2. Nulle differentias esseva trovate per chromatographia inter le polysaccharidas derivate ab differente racias.

Nulle del fractiones esseva immunogenic o toxic in administrationes subcutanee in muses. Le fractiones bacterial non absorbeva anticorpore protectori ab un sero equin anti erysipelas. Omne le fractiones esseva serologicamente active in le test de hemagglutination. Tamen, fraction C_2 esseva active con sero anti erysipelas de origine porcin solmente, durante que le altere fractiones esseva active etiam con tal sero de origine equin e de conilio. Esseva trovate que fractiones C_1 e C_{1T} contineva 1 componente a specificitate per typo e 2 componentes a specificitate per specie e isto variava ab un racia bacterial al altere. Le fractiones bacterial fixava complemento in le presentia de sero anti erysipelas ab conilios e porcos sed non ab cavallos.

The Antigenic Structure of Virulent and Avirulent Strains of *Erysipelothrix rhusiopathiae*. III. The Agar Gel Diffusion-Precipitation Test

Marian Truszczynski, Dr. Med. Vet.

SUMMARY

Type-specific and species-specific antigens were demonstrated in several strains of *Erysipelothrix rhusiopathiae* by means of the gel diffusion-precipitation test. Species-specific antigens were found in broth cultures, and in bacterial fractions C_1, Np_1, Np_2, and Np_3. Type-specific antigens were found in acid extracts prepared by the Lancefield method, in broth cultures, and in fractions C_1, C_2, Np_1, Np_2, and Np_3. The species-specific antigen of strain StG was not identical to the corresponding antigen of other strains. This may have been related to the lack of immunizing properties of strain StG. The 4 antigens found in fractions Np_1, Np_2, and Np_3 of strains B and StF were not found in other strains. Strains B and StF had excellent immunogenic properties but the other strains investigated were poor immunizing agents.

INTRODUCTION

The purpose of this work was to investigate soluble antigens, acid extracts, and bacterial fractions of several strains of *Erysipelothrix rhusiopathiae* using the agar gel diffusion-precipitation test. The relationship between the 2 serotypes, A and B, was also studied.

Materials and Methods

The technique described by Matthews[10] was used. Polyvalent anti-erysipelas horse serum[14] was the source of antibody. Forty-eight to 72-hour cultures of *Ery. rhusiopathiae* in horse-meat broth, and in horse-meat broth containing 3% normal horse serum, were used as antigens. Before use, the cultures were kept at 4 C. for 3 to 6 days to allow more antigenic material to dissolve. Acid extracts of *Ery. rhusiopathiae* were prepared according to Lancefield's method[9] and diluted 1:5. The polysaccharide, nucleoprotein, and protein fractions of strains A, B, StG, and StF, described elsewhere,[14] were also used. The following strains of *Ery. rhusiopathiae* were used: G2505, Br, B10, 1024, B9XVI,E944/XVI,* St55, St54, and St53.** The C_1, C_{1T}, C_2, Np_2, and Np_3 fractions[14] were used in a concentration of 2 mg./ml. in these experiments. The following control materials were used: polyvalent anti-erysipelas horse serum, noninoculated horse-meat broth containing 3% normal horse serum, and 0.85% NaCl solution.

Results

The interpretation of the following results was based on these postulates: (1) Each precipitation zone obtained in the agar gel precipitation test corresponds to only one antigen; (2) the fusion of 2 zones of neighboring antigen-antibody systems denotes the identity of the antigen compared; and (3) the intersection of the precipitation zones denotes the heterogeneity of the antigens compared.[11]

The results of the first experiment are demonstrated in Figure 1. Every strain belonging to serotype A or B, and cultivated in horse-meat broth containing 3% normal horse serum, produced 4 precipitation zones with polyvalent anti-erysipelas horse serum. The zones were designated for serotype A as IA, I'A, IIA, and IIIA, and for serotype B as IB, I'B, IIB, and IIIB. Zones II and III, produced by antigen from all of the 6 strains investigated, fused, thereby indicating identity. These antigens, therefore, are common to both serotypes and are species-specific antigens. The external precipitation zones IA and I'A intersect the external zones IB and

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* Prof. Dr. E. Roots, Giesen, Germany, supplied these strains.

** Staub's strains, differing in immunogenic properties, are available in the strain collection of the Veterinary Institute, Pulawy.

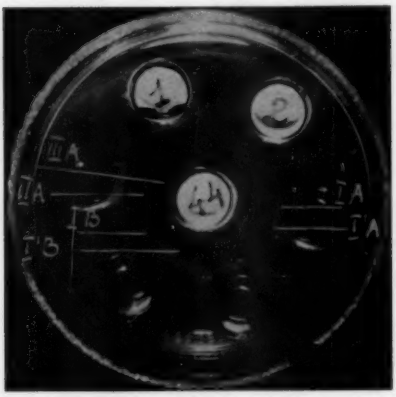

Fig. 1—Precipitation of broth cultures of 6 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) G2505, serotype A; (2) Br, serotype B; (3) B10, serotype A; (4) 1024, serotype A; (5) B9/XVI, serotype B; (6) E944/V, serotype B; central basin, anti-erysipelas horse serum.

I'B. These antigens are therefore type-specific. In the next experiment, various of Staub's strains of serotype B and one strain of serotype A were compared. The results are shown in Figure 2. In a further experiment, Staub's strains were compared with a virulent serotype B. The avirulent Staub's strain produced the same number of precipitation zones as the virulent strains. Precipitation zones I and I', characterizing the type-specific antigen, were identical with those of the virulent strain of serotype B. The zones characterizing the species-specific antigen were also identical for all strains with the exception of the StG strain. The IIG zone (zone II of strain StG) was only partially related to zone II of the remaining Staub's strains and of the A and B virulent strains, as indicated by their partial fusion (Fig. 2). The StG strain, as demonstrated in a previous paper,[7] did not immunize pigs and mice against erysipelas infection.

Soluble antigens were obtained from *Ery. rhusiopathiae* cultures in horse-meat broth without normal horse serum. It was found that the species-specific antigen passed into the liquid mediums more abundantly than did the type-specific antigens.

It may be seen from Figure 3 that the acid extracts, prepared according to Lancefield's method, contained only type-specific antigens.

The results concerning the determination of the number of precipitation zones in fractions from the A, B, StG, and StF strains are illustrated in Figures 4 to 9 and Table 1.

There were significant differences in the number of precipitation zones produced by fractions of the individual strains. The fractions of the B and StF strains produced more precipitation zones; *i.e.*, according to Ouchterlony,[11] they contained

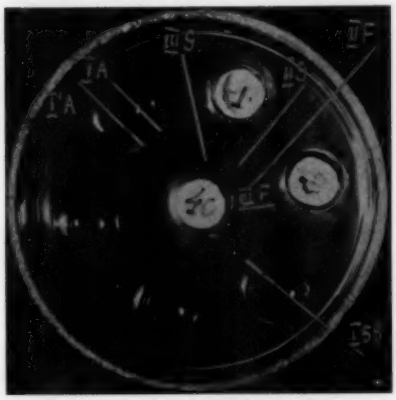

Fig. 2—Precipitation of broth cultures of 6 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) StG; (2) StF; (3) S 55; (4) St54; (5) St53; (6) A; central basin, anti-erysipelas horse serum.

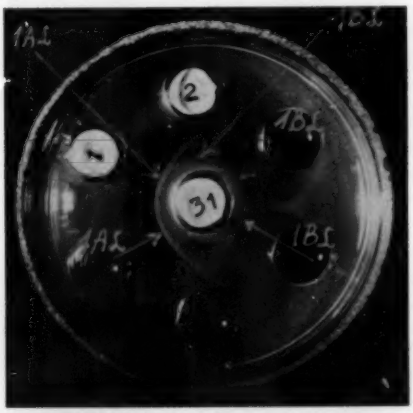

Fig. 3—Precipitation of acid extracts of 6 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) A, serotype A; (2) 944/17/XVI, serotype B; (3) StG, serotype B; (4) StF, serotype B; (6) S7, serotype A; central basin, anti-erysipelas horse serum.

TABLE 1—Zones of Precipitation Found in Various Fractions of Strains A, B, StG, and StF of Erysipelothrix rhusiopathiae

Fraction	Strain			
	A	B	StG	StF
C_1	1A*	1B**	1G**	1F**
	2A**	2B**	2G**	2F**
	3A†	3B†	3G†	3F†
C_{1T}	$1A_T$**	$1B_T$**	$1G_T$**	$1F_T$**
	$2A_T$**	$2B_T$**	$2G_T$**	$2F_T$**
	$3A_T$†	$3B_T$†	$3G_T$†	$3F_T$†
Np_1	4A**	4B	4G**	4F
	5A**	5B**	5G**	5F**
	6A†	6B	6G†	6F
	.....	7B†	.....	7F†
Np_2	.....	8B	.....	8F**
	.....	9B	.....	9F†
	.....	10B**	.....	.....
	.....	11B†	.....	.....
Np_3	.....	12B**	.....	10F
	.....	13B†	.....	11F
	.....	.....	.....	12F
	.....	.....	.....	13F**
				14F†
C_2	7A	13B	.....	15F
	8A	.....	.....	.....

* Designation of precipitation zones (*see* also Fig. 4 to 9). ** Precipitation zones corresponding to type-specific antigen. † Precipitation zones corresponding to the species-specific antigen.

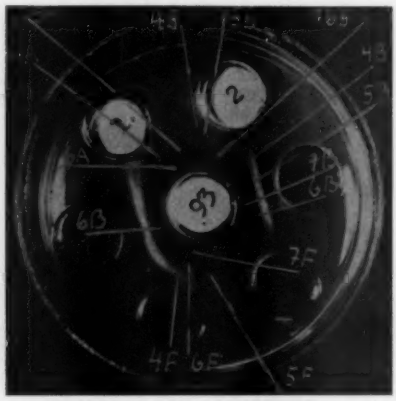

Fig. 5—Precipitation of 6 fractions from 4 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) Np_1A; (2) Np_1G; (3) Np_1B; (4) Np_1A; (5) Np_1F; (6) Np_1B; central basin, anti-erysipelas horse serum.

more antigens than did the StG and A strains.

In other experiments, the relationship between the antigens of the individual fractions was investigated. These results are also partially reported in Table 1.

The species-specific antigen common to the A and B serotypes, and A or B type-specific antigens were found in each strain tested. In the C_1 and C_{1T} fractions of the B strain, which had been classified by Dedie's method [3] as serotype B, the A type-specific antigen (Figs. 4, 6, 8, 9) was found in addition to the B type-specific antigen. The greatest quantity of these antigens was found in the polysaccharide C_1 and C_{1T} fractions. They were also found in the nucleoprotein Np_1, and protein Np_2 and Np_3 fractions. The species-specific antigen of the StG strain was partially heterolo-

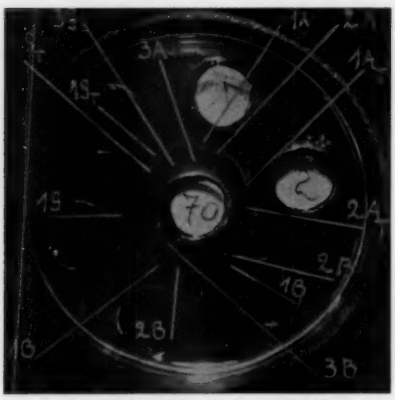

Fig. 4—Precipitation of 6 fractions from 3 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) C_1A; (2) C_1A_T; (3) C_1B; (4) C_1B_T; (5) C_1G; (6) C_1G_T; central basin, anti-erysipelas horse serum.

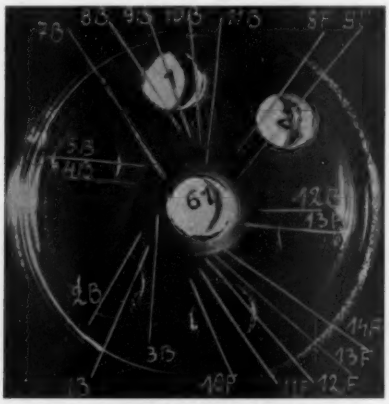

Fig. 6—Precipitation of 6 fractions from 2 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) Np_2B; (2) Np_2F; (3) Np_3B; (4) Np_2F; (5) C_1B; (6) Np_1B; central basin, anti-erysipelas horse serum.

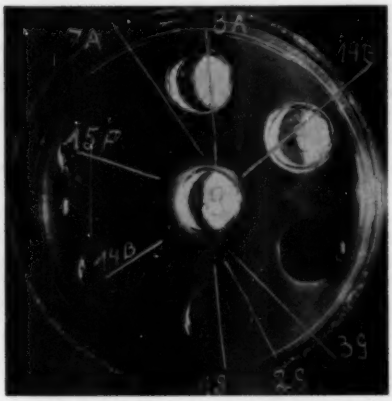

Fig. 7—Precipitation of 6 fractions from 4 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) C_2A; (2) C_2B; (3) C_2G; (4) C_1G; (5) C_1B_T; (6) C_2F; central basin, anti-erysipelas horse serum.

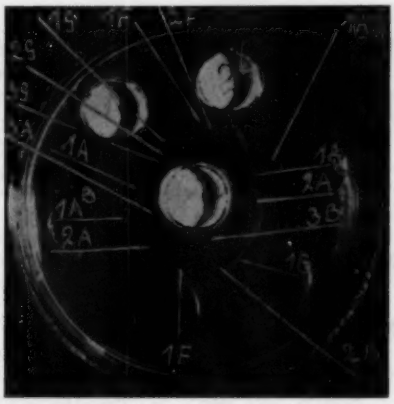

Fig. 8—Precipitation of 6 fractions from 4 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) C_1G; (2) C_1F; (3) C_1A; (4) C_1B; (5) C_1F; (6) C_1A; central basin, anti-erysipelas horse serum.

gous (a partial intersection) to the species-specific antigen of the remaining strains (A, B, StF). This was a confirmation of the previously described results with liquid broth cultures and may be related to the lack of immunogenic properties of the StG strain. Additional antigens were found in the A, B, and StF strains. These antigens were contained in the polysaccharide C_2 fractions.[2,14] Only 1 of the 2 antigens contained in the C_2 fraction of the A strain, and the C_2 antigen of the B strain, were homologous. The remaining antigens contained in the C_2 fractions seemed to be heterologous. All antigens contained in the C_2 fractions were different from those found in all of the other fractions. Additional antigens of the B and StF strains were found in isoelectric nucleoproteins (Np_1). One of these antigens was identical for both strains (4B=4F). The other was probably heterologous (6B = 6F). The chemical structure of these antigens was not investigated. In addition to the type-specific and species-specific antigens, new antigens were found in the protein fractions precipitated with the trichloracetic acid (Np_2B and Np_3B). Two of the antigens (8B and 9B) were found in the B strain, and 3 antigens (10F, 11F, 12F) were found in the StF strain. Their interrelationship was not investigated because of lack of material. It is probable that the B strain contained 2 to 4 additional antigens. Attempts to determine the exact

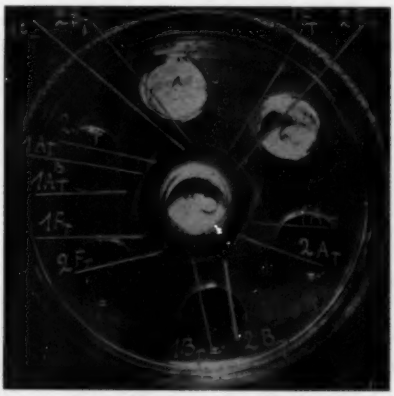

Fig. 9—Precipitation of 6 fractions from 4 strains of Erysipelothrix rhusiopathiae with anti-erysipelas horse serum. Contents of the basins: (1) C_1G_T; (2) C_1F_T; (3) C_1A_T; (4) C_1B_T; (5) C_1F_T; (6) C_1A_T; central basin, anti-erysipelas horse serum.

number of these antigens resulted in failure, since lack of material did not permit the determination of whether the 4B and 6B zones of the Np_1 fraction intersected or fused with the 8B and 9B zones of the Np_2B fraction. Of the 3 antigens of the Np_3F fraction (10F, 11F, 12F), 1 (12F) was homologous to the 6F antigen of the nucleoprotein fraction of strain StF. The StF strain had 4 different antigens contained in the protein fractions.

Discussion

Using the agar gel diffusion-precipitation test, the data of Atkinson and Collins,[1] Dedie,[3] and Roots,[12] on the presence of type-specific and species-specific antigens of *Ery. rhusiopathiae*, were confirmed. These antigens were found in liquid cultures as soluble antigens. Since in liquid cultures the species-specific antigen is found in a greater amount than the type-specific antigen, it is not surprising that filtrates of cultures of A and B serotypes of *Ery. rhusiopathiae* were precipitated both by anti-A and anti-B erysipelas serum.[4] The observation of differences between the species-specific antigen of the StG strain and that of the remaining strains of *Ery. rhusiopathiae* was of considerable interest. The StG strain, even in the living state, was not immunogenically active. Naturally, this finding is not proof of a relationship between the unique structure of the species-specific antigen of the StG strain and its lack of immunizing properties. However, Krauss *et al.*[9] demonstrated by gel precipitation that the species-specific antigen of the Swedish avirulent strain AV/R_9 is identical to the species-specific antigen of the StG strain. The same workers showed that the AV/R_9 strain, although immunogenic for mice, immunized pigs against erysipelas infection to a small degree only.

The type-specific antigen was the only one found in the acid extracts. This provided a simple and reliable test for the classification of *Ery. rhusiopathiae* in 2 different serotypes.

Gel precipitation analysis of the fractions of the A, B, StG, and StF strains revealed that the species- and type-specific antigens were localized not only in polysaccharide fractions C_1 and C_{1T}, but also in protein fractions Np_1, Np_2, and Np_3. Gralheer [7] has demonstrated that the type-specific haptens A and B have polysaccharide structure. It is possible that the Np_1, Np_2, and Np_3 fractions are not pure proteins.

The finding of more antigens in the protein fractions of the B and StF strains than in those of the StG and A strains may be related to the better immunogenic properties of the B and StF strains. This would be consistent with the statement of Traub,[13] Dedie,[4] and Joubert [8] that the immunizing antigen of *Ery. rhusiopathiae* is a protein.

It was known from previous investigations [2] that the antigen localized in the C_2 fractions was heterologous to the antigens localized in the C_1 fractions. In the present investigations, this conclusion was confirmed by means of the agar gel diffusion-precipitation test. The C_2 fractions probably contain 2 or 3 different antigens.

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SUMMARIO IN INTERLINGUA

Le Structura Antigenic de Virulente e Avirulente Racias de *Erysipelothrix rhusiopathiae*. III. Le Test de Precipitation a Diffusion in Gel de Agar

Antigenos a specificitate per typo e per specie esseva demonstrate in plure racias de *Erysipelothrix rhusiopathiae* per medio del test de precipitation a diffusion in gel de agar. Antigenos a specificitate per specie esseva trovate in ambe culturas e in le fractiones bacterial C_1, Np_1, Np_2, e Np_3. Antigenos a specificitate per typo esseva trovate in extractos acide preparate per le methodo de Lancefield in culturas a bouillon e in le fractiones C_1, C_2, Np_1, Np_2, eNp_3. Le antigeno a specificitate per specie del racia StG non esseva identic con le correspondente antigeno de altere racias. Isto esseva possibilemente relationate con le manco de proprietates immunisante del racia StG. Le 4 antigenos trovate in le fractiones Np_1, Np_2, e Np_3 del racias B e StF non esseva trovate in altere racias. Racias B e StF habeva excellente proprietates immunogenic, sed le altere racias investigate esseva inefficace agentes de immunisation.

Studies of Bovine Enteroviruses

Carlo Moscovici, Ph.D.; Michele LaPlaca, M.D.; John Maisel, M.D.; C. Henry Kempe, M.D.

SUMMARY

Eleven strains of enteric viruses of bovine origin were isolated and characterized. Attemps were made to relate these new isolates to currently known bovine enteroviruses. Early experience suggested that, in addition to conventional techniques, purification of crude isolates by cellulose resin ion exchange columns may be of value in the eventual classification of enteroviruses of bovine origin.

INTRODUCTION

The alimentary tracts of some domestic and nondomestic animals have proved to be an excellent reservoir for cytopathogenic agents. A number of viral agents have been isolated in recent years from the feces of swine,[1,14,22,31] monkeys,[6,9,10] and cattle.[13,18] Some of these agents were isolated from sick and others from apparently healthy animals. There are many examples which indicate that viruses of bovine origin might be implicated in diseases of man.[12,26,28]

This study reports the results of the isolation of 11 viral agents from the intestinal tracts of apparently healthy newborn calves. The studies were undertaken in order to characterize and possibly to classify viral agents found in the intestinal tracts of calves and to determine their possible association with known animal and human enteroviruses. The study also sought to determine the presence or absence of antibodies against such viral agents in animal and human serum. Finally, preliminary data on ion exchange purification of one strain of bovine virus will be described.

Materials and Methods

Rectal swabs were collected from newborn calves during the fall of 1957 through the summer of 1958 by means of sterile cotton-tipped applicators. The animals were housed in the same pen during the experiment. Rectal swabs were obtained from calves each month, and new calves were added to the study as they became available. Two hundred and seventy-two fecal samples obtained from 91 calves were tested. All animals came from a dairy farm located 25 miles west of Denver. Veterinary examination confirmed the fact that none of the animals became ill during the course of the study. Rhesus monkey kidney (MK), bovine kidney (BK), guinea pig kidney (GPK), rabbit kidney (RK), and HeLa cells were used for isolation and passage. For monkey cells, the growth medium consisted of 0.5% lactalbumin hydrolysate in Earle's salt solution plus 10% calf serum. When confluency of the cell sheet was obtained, the growth medium was completely removed and 2 ml. of Earle's salt solution with 0.5% lactalbumin hydrolysate was added as a maintenance medium. Bovine kidney cells were processed in the same way, the serum component consisting of 10% fetal calf serum. Guinea pig and rabbit kidney cells were processed as previously described [23] and grown in synthetic mixture 199 (purchased from Microbiological Associates)[23] plus 10% calf serum. Growth medium was always removed and replaced with 2 ml. of Earle's salt solution with 0.5% lactalbumin hydrolysate. HeLa cells [29] were grown on a medium consisting of 199 plus 10% calf serum. Before inoculation with virus, the growth medium was removed and replaced with 2 ml. of a mixture consisting of 98.5% of medium 199, 0.5% lactalbumin hydro-

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From the Department of Pediatrics (Moscovici, assistant professor, and Kempe, professor and chairman), School of Medicine, University of Colorado, Denver. Dr. LaPlaca is a fellow of the Italian Research Council, and Dr. Maisel is a fellow of the National Foundation.

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Monkey kidney cells (Rhesus) were obtained from Shamrock Farms, Middletown, N.Y.; bovine kidney cells from Colorado Serum Co., Denver, Colo.; HeLa cells from Microbiological Associates, Bethesda, Md. The authors thank the following colleagues for supplying serum used in this study: Dr. Herbert Wenner, Kansas City, Kan., ECHO and polio antiserum; Dr. Albert Sabin, Cincinnati, Ohio, Reovirus types 2 and 3; Dr. T. L. Chow, Fort Collins, Colo., infectious bovine rhinotracheitis rabbit antiserum; Mrs. M. McDonald, Madison, Wisc., calf enteritis antiserum and vesicular stomatitis antiserum; Dr. C. M. Kunin, Boston, Mass., ECBO LCR4 rabbit antiserum; Dr. T. Moll, Pullman, Wash., rabbit antiserums against enteric viruses, BE-1, BE-239, BE-240a, and BE-180; Dr. H. Malherbe, Johannesburg, S. A., antiserums against SA3 and SA7. Erythrocytes were obtained from Dr. A. Schwarz, Pitman-Moore Co., Indianapolis, Ind., and Dr. R. Hull, Eli Lilly Co., Indianapolis, Ind.

Virus strains were obtained from Dr. T. Moll, Pullman, Wash., strains BE-1 and BE-180 in bovine kidney cells of unknown passage number; Dr. Herbert Wenner, Kansas City, Kan., Reovirus type 1 (Lang) in the 4th monkey kidney passage; Dr. Albert Sabin, Cincinnati, Ohio, Reovirus type 2 (Jones) and Reovirus type 3 (Dearing) in a 9th monkey kidney passage; Dr. C. M. Kunin, Boston, Mass., strain LCR4 in its 1st passage, in bovine kidney after 6 passages in steer kidney and 4 passages in monkey kidney cells.

lysate in Earle's salt solution, and 1% proteose peptone No. 3 (Difco).

Serum.—Individual human serum obtained from residents of the State Home and Training School were kept at −20 C. until used. The dairy farm where the calves were housed is part of this institution.

Animals.—Suckling mice, 48 hours old, were inoculated by intracerebral and intraperitoneal routes. Three blind passages were made from pools of brain and leg muscles obtained 6 days after the inoculation. The mice were examined daily during a 3-week period of observation. Weaned mice[20] were given intramuscular inoculations of 2.5 mg. of cortisone acetate on 3 consecutive days. On the 4th day, the mice were inoculated by intracerebral and intraperitoneal routes with undiluted fluid containing virus. On the 6th and 12th days after infection, mouse brain suspensions were reinoculated into monkey and bovine kidney cells. Adult mice were inoculated by both intracerebral and intraperitoneal routes without any previous cortisone treatment.

Neutralization Tests.—These tests were performed in MK and BK cell cultures with 100 TCD_{50} of virus, determined by titration in tissue culture; endpoints were obtained by the method of Reed and Muench.[25] Neutralization tests were performed by mixing virus suspension with an equal amount of antiserum, using a standard method.[3] Following an hour of incubation at room temperature, 0.2-ml. aliquots of the serum-virus mixture were added to tubes of MK or BK cultures. All tubes were incubated at 37 C. and examined every 2 days until the 8th day. Control virus titrations were carried out simultaneously. The serum titer was recorded as the highest final dilution of serum which prevented the appearance of a cytopathic effect (CPE). All the serums used in the tests were inactivated at 60 C. for 30 minutes.

Erythrocytes.—Horse, sheep, human "O", swine, chicken, guinea pig, bovine, rhesus monkey, and cynomologus monkey erythrocytes were utilized. Mouse erythrocytes were tested[26] to determine if any hemagglutinins or hemagglutinating properties were present in the tissue culture fluids after CPE had occurred. A cell concentration of 0.5% in buffered saline solution was utilized, except for the mouse red blood cells which were used for the concentration of 1.0%. Hemagglutination was performed at room temperature and in the refrigerator at 5 C. Results were read after an hour at room temperature, and after 15 minutes when hemagglutination was performed in the cold. Elution followed rapidly after hemagglutination occurred, with the exception of human "O" erythrocytes.

Plaque Purification.—The method developed by Dulbecco,[5] with slight modification,[16] was used in order to purify virus isolates in MK and BK cells from the original material. Each petri plate culture was inoculated with 0.3 ml. of the appropriate virus dilution; the adsorption time was 1 hour at 37 C. The cultures were not washed postadsorption. Six milliliters of an agar overlay was then added. Plaques were visible after 4 to 5 days upon adding a 2nd agar overlay with neutral red (3 ml./petri dish, consisting of 0.01% neutral red and 0.9% agar). Each virus was purified 3 times by picking a single plaque each time.

Histology.—Histologic examinations were made according to the procedure described by Enders and Peebles.[6] Control tubes and those with early CPE were fixed with Bouin's fixative for 2 hours, embedded in 4% of collodion, and stained with hematoxylin and eosin.

Preparation of the Suspected Material for Virus Isolation.—The swabs obtained from each calf were put into a tube containing 2.5 ml. of Hanks' solution with 100 units of penicillin and 0.1 mg. of streptomycin per milliliter. The tubes containing the swabs were stored in a dry-ice chest at about −70 C. When the specimens were to be used, they were thawed with warm water; the swabs were squeezed against the sides of the tube and discarded. The extracts of the swabs were then spun at 3,000 r.p.m. for 30 minutes in the cold room at 5 C. The resulting supernatant material was then harvested and used for virus isolation. The inoculated tissue culture tubes were observed daily for 14 days. As soon as CPE was noticed, a passage was made by transferring 0.2 ml. of fluid to a fresh tissue culture. No blind passages were made from tubes considered negative. Reisolation from the original material in a different batch of MK was performed to exclude the possibility of having recovered monkey virus contaminants.

Preparation of Antiserum.—Immune rabbit serum was produced separately against each prototype of bovine virus grown in MK and BK, respectively. Three 2-ml. intravenous injections of infected fluid were administered to each rabbit at 10-day intervals. A 4th intravenous injection was given 15 days after the 3rd one. Samples of serum were taken 10 days after the 4th injection and tested for the presence of neutralizing antibodies. All serum was inactivated at 60 C. for 30 minutes before each test.

Results

Isolation of Viruses in Tissue Culture.—Eleven cytopathogenic agents were isolated from 272 swab specimens obtained from 91 animals. Nine agents were cytopathogenic for MK and BK cells, while 2 affected MK cells only. Repeated passages of these 2 agents from MK to BK cells failed to result in demonstrable CPE by these 2 agents. Different MK cells were used to reisolate the same agents from the original material; these reisolates again failed to cause CPE in BK cells. All agents were passed 4 or 5 times consecutively in MK and BK tissue cultures. All successful isolations were made only during the months of June and July of 1958. None of the calves remained

carriers of these agents, since successive swabs obtained at monthly intervals were negative for virus.

Titers were quite similar in both cell lines (Table 1). Relatively low titers were

TABLE 1—Tissue Culture Titers of Bovine Enteroviruses

Isolate No.	Monkey kidney culture	Bovine kidney culture
48/4	2.2*	.....
61/4	6.0	6.5*
62/4	6.0	7.3
64/4	5.5	6.5
71/2	5.8	6.2
72/3	6.0	6.5
74/2	6.5	6.6
75/2	1.5	.....
76/2	7.0	7.2
79/2	5.5	7.0
87/1	6.8	7.0
Strain		
Moll/BE-1	6.5	6.5
Moll/BE-180	6.5	6.5
Kunin/LCR4	6.8	7.0
IBR	.....	7.0

* Expressed as the negative log of the TCD_{50} per 0.2 ml. inoculum.

obtained for isolates 48/4 and 75/2, which failed to grow in BK cells.

Characteristics of Lesions Induced in Tissue Cultures and Host Range.—The CPE of these agents in MK cells was similar to that described by Melnick [17] for the human enterovirus group, with the exception of isolates 48/4 and 75/2 which caused complete destruction of the cell sheet after 7 to 8 days from the date of inoculation. The cytopathic changes produced by isolate 48/4 were similar to those described for

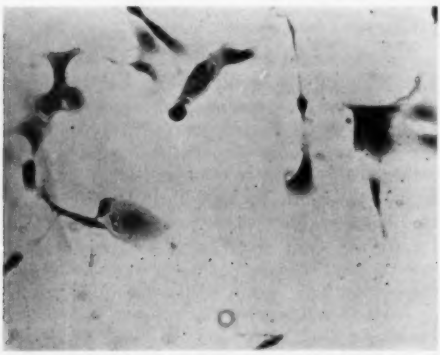

Fig. 1—Different aspects of lesions produced by isolate 48/4 in monkey kidney cells, and cytoplasmic inclusion bodies. H & E stain; x 200.

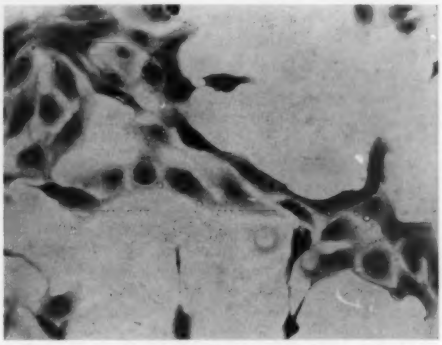

Fig. 2—Cytoplasmic inclusion bodies produced by strain 48/4 in monkey kidney cells. H & E stain; x 200.

ECHO 10,[4,30] now designated as Reovirus group.[28] Cells infected with 48/4, subsequently fixed and stained, were shown to contain homogeneous eosinophilic masses in the cytoplasm. These masses were irregular, sometimes surrounding the nucleus and at other times spreading diffusely through the cytoplasm (Fig. 1–4).

Isolate 75/2 was less rapid in its CPE and, in a stained preparation, its nuclear inclusions were distinctive (Fig. 5). These lesions resemble those described by Malherbe as the SA7 strain isolated from uninoculated vervet monkey kidney cells.[15] However, SA7 antiserum failed to neutralize

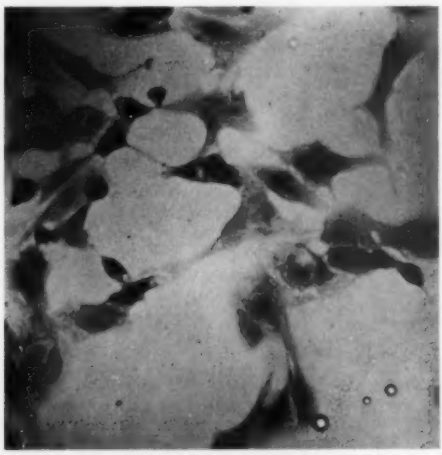

Fig. 3—Cytoplasmic inclusion bodies produced by isolate 48/4 in monkey kidney cells. H & E stain; x 280.

almost 100 TCD_{50} of isolate 75/2, and the nature of this isolate, therefore, remains as yet undetermined.

Infected fluids derived from MK and from BK cells were subsequently passed in other cell systems, including guinea pig and rabbit kidney cells and HeLa cells. At least 3 consecutive passages were made, regardless of whether CPE occurred. The observation time of infected tubes was 10 days. In order to be certain that the CPE was really due to virus effect, each 3rd passage was repassed once in MK and in BK. No CPE for MK or BK was observed from the isolates which in the table are listed as negative. The results of the spectrum of susceptibility are shown (Table 2).

It is interesting to observe that the use of GPK cells for one isolate (61/4) yielded CPE from the BK cells, but not from the MK passage. Furthermore, when HeLa cells were used, at least 3 isolates (61/4, 72/3,

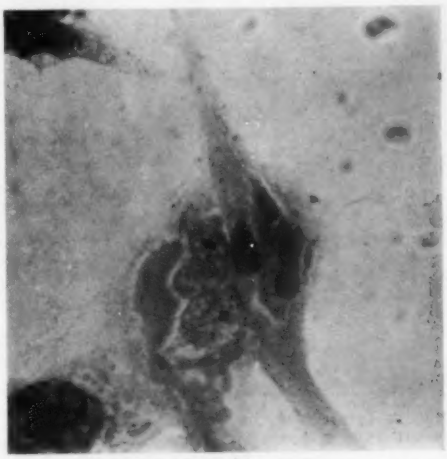

Fig. 4—Cytoplasmic inclusion bodies produced by isolate 48/4 in monkey kidney cells. H & E stain; x 520.

TABLE 2—Susceptibility of Different Cell Lines to Bovine Enteroviruses

Isolate	Monkey kidney from original material	Bovine kidney from original material	Guinea pig kidney	HeLa	Rabbit kidney	Type of cytopathic effect
48/4A	+	...	−	−	−	Slow in MK, Reovirus-like in MK,
48/4B	...	−	−	−	−	cytoplasmic inclusion bodies.
61/4A	+	...	−	−	−	Rapid in MK.
61/4B	...	+	+	+	−	Rapid in BK, GPK, and HeLa.
62/4A	+	...	+	+	−	Rapid in MK, GPK, and HeLa.
62/4B	...	+	+	+	−	Rapid in BK, GPK, and HeLa.
64/4A	+	...	+	+	−	Rapid in MK, GPK, and HeLa.
64/4B	...	+	+	+	−	Rapid in BK, GPK, and HeLa.
71/2A	+	...	n.t.	+	−	Rapid in MK and HeLa.
71/2B	...	+	+	+	−	Rapid in BK, GPK, and HeLa.
72/3A	+	...	n.t.	+	−	Rapid in MK and HeLa.
72/3B	...	+	+	−	−	Rapid in BK and GPK.
74/2A	+	...	+	−	−	Rapid in MK and GPK.
74/2B	...	+	+	+	n.t.	Rapid in BK, GPK, and HeLa.
75/2A	+	...	−	−	−	Slow in MK, intranuclear inclusion bodies.
75/2B	...	−	−	−	−	
76/2A	+	...	+	+	−	Rapid in MK, GPK, and HeLa.
76/2B	...	+	+	+	−	Rapid in BK, GPK, and HeLa.
79/2A	+	...	−	−	−	Rapid in MK.
79/2B	...	+	−	−	−	Rapid in BK.
87/1A	+	...	−	−	−	Rapid in MK.
87/1B	...	+	−	−	−	Rapid in BK.
Strain						
LCR4 (Kunin)	+	+	+	+	+	Rapid in MK, BK, GPK, Hela, and RK.
SK6/MK4/BK1						
BE-180/BK! (Moll)	+	+	+	+	+	Rapid in MK, BK, GPK, Hela, and RK.

A = from monkey kidney only; B = from bovine kidney only; + = positive; − = negative; n.t. = not tested; MK = monkey kidney; BK = bovine kidney; GPK = guinea pig kidney; RK = rabbit kidney; SK = steer kidney

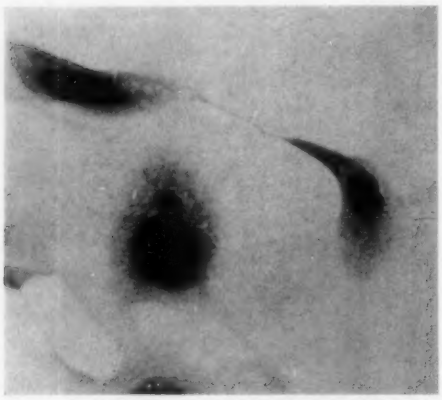

Fig. 5—Intranuclear inclusion bodies produced by isolate 75/2 in monkey kidney cells. H & E stain; x 433.

74/2) behaved in the same manner, with the exception of isolate 72/3 in which the BK passage failed to multiply in HeLa cells. In addition, the LCR4 strain of Kunin was found to be cytopathic for the strain of HeLa used in our laboratory.

Inoculation in Experimental Animals.—All 11 isolates from MK and from BK fluids were injected into suckling mice by the intraperitoneal and intracerebral routes but failed to produce disease. Weaned mice, pretreated with a cortisone acetate, were also inoculated by the 2 routes with infected fluids derived from MK and BK cells, utilizing 5 animals for each isolate and controls. None of the animals had any signs of illness. Finally, adult mice were also shown to be unaffected by these agents.

Hemagglutination.—In an attempt to facilitate the classification of the isolated viruses, hemagglutination with different types of erythrocytes was attempted. Table 3 shows the results when early virus passage material from MK and BK cells and passage material from triple plaque-purified viruses in MK and BK cells were tested.

Only isolate 48/4 hemagglutinated human erythrocytes. Inconsistent results were obtained with bovine red blood cells when repeated tests were performed with erythrocytes from different cows. In isolate 64/4, hemagglutination for guinea pig red blood cells was negative when the infected crude material was passed in BK cells, while hemagglutination was positive when the virus was grown in MK cells only.

TABLE 3—Hemagglutination Patterns with Different Erythrocytes at 5 C.

Isolate	Crude virus population								Purified virus PP 3							
	A				B				A				B			
	Hum. "O"	Bov.	G.P.	Mouse	Hum. "O"	Bov.	G.P.	Mouse	Hum. "O"	Bov.	G.P.	Mouse	Hum. "O"	Bov.	G.P.	Mouse
48/4	+	±	—	—	—	—	—	—	...	...	...	...	...	...	...	...
61/4	—	±	—	—	—	—	—	—	...	...	...	...	—	—	—	—
62/4	—	—	+	—	—	—	+	—	—	—	+	—	—	—	+	—
64/4	—	—	+	—	—	—	—	—	—	—	+	—	—	—	+	—
71/2	—	±	—	—	—	—	+	—	—	—	—	—	—	—	—	—
72/3	—	±	—	—	—	—	+	—	—	—	—	—	—	—	—	—
74/2	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
75/2	—	±	—	—	—	—	—	—	...	...	...	...	...	...	...	...
76/2	—	—	+	—	—	—	+	—	—	—	+	—	—	—	+	—
79/2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
87/1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

A = from monkey kidney only; B = from bovine kidney only; G.P. = guinea pig erythrocytes; — = no hemagglutination at 1:5 virus dilution; + = presence of hemagglutination at 1:5 or more virus dilution; ± = hemagglutination was inconsistent.

TABLE 4—Average Diameter (mm.) and Morphologic Characteristics of the Virus Plaques in Monkey Kidney and Bovine Kidney Cells

Isolate	Appearance of plaques (day)	Plaque size (mm.)	Average plaque diameter (mm.) 6th day (last day reading)	Plaque morphology
61/4A	..	.....	.....	No plaque obtained in monkey kidney.
61/4B	5	6–7	9.0–8.0	Large, circular, sharp edges, clear centers.
62/4A	4	3–4	3.5–4.5	Medium, circular, slight irregular edges, clear centers.
62/4B	5	6–7	7.5–8.0	Large, circular, sharp edges, clear centers.
64/4A	4	3–4	5.0–6.0	Medium, circular, slight irregular edges, clear centers.
64/4B	4	5–6	8.0–9.0	Large, circular, sharp edges, clear centers.
71/2A	4	2–3	2.5–3.5	Small, circular, indented edges, hazy centers.
71/2B	5	**	3.5–4.0	Medium, circular, indented edges, centers sometimes clear.
72/3A	4	4–5	5.0–6.0	Medium, circular, indented edges, clear centers.
72/3B	4	7–8	9.0–10.0	Large, circular, indented edges, clear centers.
74/2A	5	3–4	3.5–4.0	Medium, circular, indented edges, hazy centers.
74/2B	5	1–2	1.5–2.5	Small, circular, very irregular edges.
76/2A	4	3–4	5.0–6.0	Medium, circular, slight irregular edges, clear centers.
76/2B	4	6–7	8.0–9.0	Large, circular, indented edges, clear centers.
79/2A	5*	.....	8.0–9.0; 4.0–5.0; 1.0	Large, medium, and small plaques, all with diffuse boundaries.
79/2B	5	7–8	8.5–9.0	Large, circular, indented edges, clear centers.
87/1A	5	3–4	5.0	Medium, circular, indented edges, centers not very clear.
87/1B	5	3–4	5.0	Medium, circular, indented edges, hazy centers.
STRAIN LCR4 B (Kunin)	5	10	15.0	Very large, circular, sharp edges, clear centers.

A = in monkey kidney only; B = in bovine kidney only; * Three different plaques were observed on PP1 from MK_8 (recloning is under study). ** Plaques were not clear enough for measurement.

Similar differences were recorded for isolates 71/2, 72/3 and 74/2. However, when the progeny from each of these 3 agents were subjected to plaque-purification, such purified isolates invariably had hemagglutination patterns which were similar, regardless of whether MK- or BK-infected fluids were tested.

Plaque Production with Bovine Enteroviruses.—Each viral agent was purified 3 times in a virus stock prepared from the 3rd plaque-purified clone in MK and BK cells. Typical plaques are shown (Fig. 6–8) and their characteristics are described (Table 4).

Plaques were not obtained from isolates 48/4 and 75/2. Furthermore, plaques could not be obtained from isolate 61/4 when MK cells were used. For the most part, BK cell plaques were larger, more nearly circular, and had clearer centers than plaques produced in MK.

There were 2 antigenically distinct groups among the 6 agents isolated (Table 5).

The antigenic relationship between isolate 48/4 and the Reovirus group (ECHO

TABLE 5—Antigenic Relationship of Bovine Enteroviruses as Revealed by Cross-Neutralization Test*

Isolate	Immune serum					
	62/4	64/4	76/2	74/2	79/2	87/1
62/4	200**	200	200	0	10	0
64/4	400	250	400	0	0	0
76/2	100	100	100	0	0	0
74/2	10	10	0	100	100	200
79/2	0†	20	0	200	4,000	200
87/1	0	0	0	100	400	100

* All the neutralization tests were made in monkey kidney cells and hyperimmune serum was obtained from rabbits inoculated with undiluted fluid containing virus grown in monkey kidney cells only. ** Highest dilution of serum neutralizing 100 TCD_{50} of virus. † Negative at dilution 1:10.

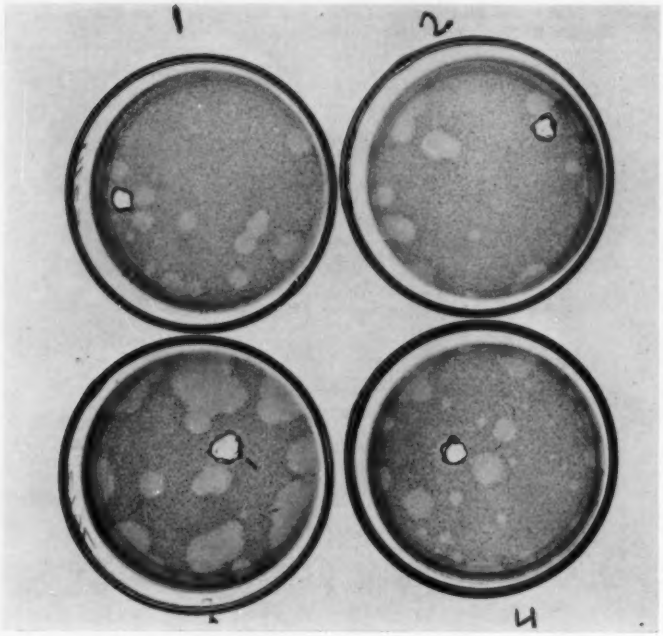

Fig. 6—Plaques resulting from the inoculation of 4 enteroviruses into monkey kidney cells. Plate 4 shows different types of plaques. Plates were read 4 days after infection (isolates 62/4, 64/4, 76/2, and 79/2).

10), using 2 different serologic techniques, is shown (Table 5, 6, 7).

Both tests showed the serologic relationship between 48/4 and the Reovirus group. This relationship, however, was more evident when the hemagglutination-inhibition test was used. No antigenic relation was ever observed between isolate 48/4 and Malherbe SA3 strain.

Comparative Studies with Other Bovine Enteroviruses.—Recently, viral agents have been isolated from calves by Kunin and Minuse [13] and by Moll *et al.*[18, 20] The results of cross-neutralization tests for a variety of bovine enteroviruses are shown (Table 8).

From these results, there is an indication of antigenic similarity between the 2 Moll's strains and strain LCR 4 (Kunin). One of

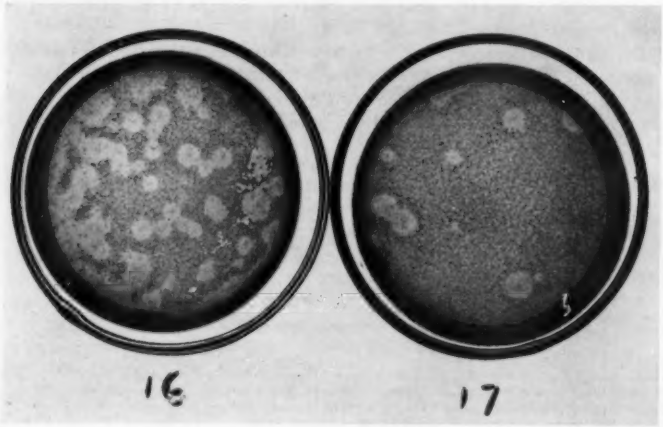

Fig. 7—Plaques resulting from the inoculation of another bovine isolate into monkey kidney cells. Notice the appearance of hazy centers. Plates were read after 4 days (isolate 74/2).

TABLE 6—Antigenic Relationships Between Isolate 48/4 and the Reovirus Group as Revealed by Cross-Neutralization Test

Virus	Immune serum					
	48/4	Reovirus type 1	Reovirus type 2	Reovirus type 3	SA3*	SA7*
48/4	1,000**	40	0	0	0	0
Reovirus type 1	0	160	NT	NT	40	0
Reovirus type 2	0	NT	160	NT	0	0
Reovirus type 3	0	NT	NT	160	0	0

Obtained from Malherbe.[15] ** Serum dilution used to neutralize 100 TCD_{50} of virus. NT = not tested.

our prototypes (64/4) appeared to be related to bovine enteroviruses described by Moll and Kunin, but none of the other isolates belonging to our 2nd group (74/2, 79/2, and 87/1; *see* Discussion) were found to be related. Serum prepared against vesicular stomatitis (Indiana and Georgia) and against calf pneumonia enteritis viruses failed to neutralize our bovine isolates.

Neutralizing Antibody Studies in Animals and Human Serum.—Neutralizing titers of serum of calves from which bovine isolates were obtained are shown (Table 9). Bovine strains were not always neutralized by their homologous calf serum, while some were neutralized by other serum samples from the group.

Table 10 shows the presence of neutralizing antibodies against bovine isolates in different animal serum samples.

Table 11 shows the presence of neutralizing antibodies against bovine isolates in human serum samples.

TABLE 7—Hemagglutination-Inhibition Titers of 5 Representative Rabbit and Monkey Serums Against Isolate 48/4 and Reovirus Strains

Viruses	Representative serum*				
	48/4 (rabbit)	Reovirus type 1 (rabbit)	Reovirus type 2 (monkey)	Reovirus type 3 (rabbit)	SA3 Malherbe (rabbit)
48/4	1:1,280	1:160	1:320	0	0
Reovirus type 1	1:1,280	1:320	1:640	1:640	1:640
Reovirus type 2	1:1,280	1:160	1:640	1:320	1:80
Reovirus type 3	1:1,280	1:160	1:320	1:640	1:320

* All the immune serums were adsorbed with human group "O" erythrocytes and with Kaolin prior to their use in hemagglutination-inhibition tests according to the procedure described by Rosen.[27]

Preliminary Studies on Virus Purification by Chromatography of N,N-diethyl-aminoethyl-cellulose Column.*—Recent studies by Hoyer *et al.*[7] suggested that both N,N-diethyl-aminoethyl-cellulose (DEAE) and Ecteola-cellulose anion exchangers can be utilized for improved purification of a number of animal viruses. Isolate 62/4, in its 4th BK passage, was utilized for this

* Technical assistance by Dr. David Love is acknowledged.

TABLE 8—Comparison by Cross-Neutralization Test of the Antigenic Properties of Bovine Enteroviruses from Various Sources

Virus	Immune serum*						
	BE 1	BE 180	64/4	LCR 4	IBR	BE 239	BE 240a
BE 1 (Moll	400**	200	200	200	NT	100	0
BE 180 (Moll)	100	200	200	150	NT	0	0
64/4† (Moscovici)	100	0	250	100	50	0	0
LCR 4 (Kunin)	0	250	250	300	NT	0	0
IBR‡	0	0	0	0	800	0	0

* Prepared in rabbits. ** Highest dilution of serum neutralizing 100 TCD_{50} of virus. † Prototype of the group which includes strains 62/4, 64/4, and 76/2. ‡ The neutralization tests for IBR were all done in bovine kidney cells. NT = Not tested.

Fig. 8—Isolate 79/2 in monkey kidney cells (same as Figure 6, plate 4). It shows 3 different types of plaques in a higher magnification. Read after 5 days.

experiment. Crude viral suspension, after centrifugation at 4,000 r.p.m. for 30 minutes, was run onto a 1 x 15-cm. column packed with DEAE and eluted with an increasing salt gradient held at a constant pH with phosphate buffer, pH 7.2. Following elution, all tubes (5 ml. of eluate each) were read at 260 and 280 mμ. Each tube was then tested for infectivity by inoculating BK cells. Results of a typical experiment are shown (Fig. 9). The 2 arrows indicate presence of active virus (tubes 36 and 136). The 2 peaks were separately replaqued, and 2 distinct types of plaques were obtained. The 1st peak resulted in a very large plaque, while the 2nd peak resulted in a small plaque. The progeny from the large plaque hemagglutinated guinea pig red blood cells, whereas the progeny from the small plaque did not. As indicated (Table 3), isolate 62/4 in the crude form or in the plaque-purified form

TABLE 9—Neutralizing Titers in Serum of Calves from which Bovine Enterovirus Isolates were Obtained

Bovine isolates	62/4	64/4	76/2	74/2	79/2	87/1	61/4	71/2	72/3
62/4	0	50	50	0	0	0	0	50	0
64/4	100	50	100	0	0	0	0	0	50
76/2	10	10	50	50	100	100	0	0	0
74/2	0	50	0	0	0	0	10	50	0
79/2	0	0	10	100	0	0	0	50	50
87/1	0	0	100	50	100	100	10	0	0
61/4	0	0	0	0	0	0	100	0	0
71/2	50	50	0	0	0	0	50	0	0
72/3	0	0	0	50	100	0	0	0	0

TABLE 10—Presence of Bovine Enteroviruses Antibodies in Different Animal Species

Virus tested	No. serums tested for each species	Species (No. with antibodies)				
		Cattle	Monkey	Horse	Swine	Sheep
48/4	15	15	7	....	....	....
61/4	15	4	6	....	....	....
74/2	15	9	3	....	....	....
87/1	15	7	..	....	....	....

TABLE 11—Presence of Bovine Enteroviruses Antibodies in Human Serum

Virus tested	No. serums tested	No. serums with antibodies*
62/4	24	2
79/2	24	24
87/1	24	24

* Neutralization titer expressed as 1:20 to 1:40 or greater.

was able to hemagglutinate guinea pig erythrocytes.

Discussion

Classification and characterization of currently available bovine enteroviruses are in a condition as chaotic as that which existed for the ECHO viruses a short time ago. As has been suggested by Kalter,[11] reference serum and prototypic viruses will be essential for the successful completion of classification and for interrelationship studies.

Among our 11 isolates, some fairly obvious groupings emerged. Isolate 48/4 might be proposed as Reovirus type 4. This isolate has properties common with other members of the Reovirus group, as shown by cytoplasmic inclusions (Fig. 1–4), ability to hemagglutinate human "O" erythrocytes, lack of CPE in HeLa cells, no plaque production, and by serologic tests. However, it could not be adapted to other cell lines such as BK or GPK cells,[8] while Reovirus strains are adapted with relative ease.[24] That this strain was indeed of bovine origin was demonstrated by 3 consecutive reisolations in MK cells with the original material. Moreover, the SA3 antiserum derived from a strain of Reovirus of simian origin[15] was not related to isolate 48/4 either by neutralization or by inhibition-hemagglutination tests. The SA3 strain was neutralized by Reovirus antiserum type 1. Isolate 75/2, which produced intranuclear inclusion bodies, was not serologically related to any of the other isolates.

Among the other 9 isolates, 6 have been studied so far by cross neutralization. They formed 2 distinct serologic groups. The 1st included isolates 62/4, 64/4, and 76/2. All of these viruses hemagglutinated guinea pig and monkey erythrocytes. While the titer of guinea pig erythrocytes was used in the order of 1:40 titers, monkey erythrocytes were consistently higher than 1:160, regardless of whether blood cells from macacus or rhesus monkeys were employed. Isolate 64/4, chosen as prototype, has a serologic relationship with bovine viruses described by Moll and Kunin. Comparative studies with known bovine strains suggest that Moll isolates BE-1 and BE-180 should be considered as belonging to the same serologic group,[19] and that strain LCR4 (Kunin) is closely related to Moll's group.[18]

The 2nd group included isolates 74/2, 79/2, and 87/1. These isolates form a new serologic group because they are not related to any known bovine enteroviruses. While these isolates were antigenically related, strain 74/2 did have a cytopathic spectrum which differs from its group companions (Table 2). Moreover, strain 74/2, when purified in MK cells only, had a clone which was able to hemagglutinate mouse erythrocytes at a high titer. Isolates 61/4, 71/2, and 72/3 are still under study.

Marked differences in properties among the same virus isolate or between serologically related isolates have been demon-

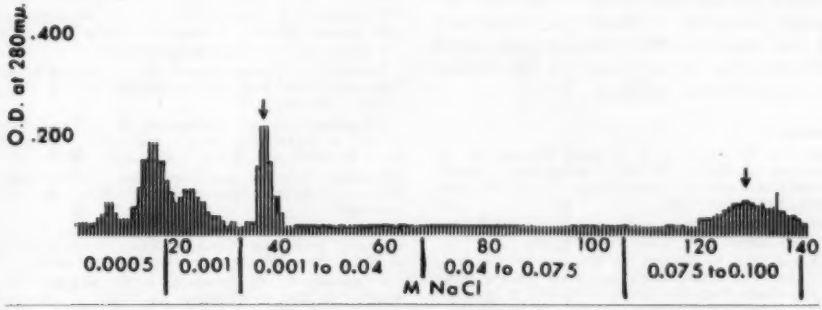

Fig. 9—Separation of 2 infectious fractions from bovine virus isolate 62/4.

strated, depending upon whether such isolates were studied in MK or BK cells. For example, isolate 61/4 had a marked difference in CPE, depending upon whether it was passed in MK or in BK cells. Infectious material grown in MK only could be repeatedly passed in GPK, RK, and HeLa cells without developing any CPE, while the same strain grown in BK cells only could be passed in GPK and HeLa cells with definite CPE being observed, and RK cells remained unsusceptible (Table 2). Similar phenomena were shown for isolate 72/3 and 74/2 when passed in HeLa cells (Table 2). Further, when isolate 74/2 was purified in MK cells only, its progeny hemagglutinated mouse erythrocytes. Finally, when isolate 79/2 was purified in MK cells only, 3 different plaque sizes were detected, but when the same strain was purified in BK cells only, 1 large plaque was consistently observed.

The role these bovine viruses might have in animal pathology is unknown. However, neutralizing antibodies against some of our isolates were found in several cows and monkeys but not in sheep, horses, and swine.

Serologic data obtained from 24 human serum samples tested against 3 of our isolates suggests the possibility that human infection occurred by these bovine viruses. These human serum samples were obtained from young adults working in the dairy farm in close contact with the animals from which our isolates were obtained.

The present confusion existing in the classification of bovine enteroviruses is the result of the highly mixed viral population found in our isolates. Different cell lines can act as selectant for different characteristics of respective isolates. The approach suggested by Hoyer *et al.*,[7] utilizing the pattern of elution of a given virus by purification of the isolates by cellulose resin ion exchange columns, suggests one approach which has considerable promise and which would probably be superior to the classic plaque-purification method.

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SUMMARIO IN INTERLINGUA

Studios de Enteroviruses Bovin

Dece-un racias de virus enteric de origine bovin esseva isolate e characterisate. Esseva interprendite le essayo de relationar iste nove isolatos con currentemente cognoscite enteroviruses bovin. Le prime experientias suggere que—a parte le technicas conventional—le purification del isolatos crude per medio de columnas de excambio de iones a resina cellulosic es possibilemente de valor in le classification de enteroviruses de origine bovin.

Antibiotic Sensitivity of Bovine Vibrios

W. N. Plastridge, Ph.D., and M. E. Koths, B.S.

SUMMARY

For the purpose of determining the effect of the medium on the antibiotic sensitivity of *Vibrio fetus* and comparing the antibiotic sensitivity of *V. fetus* and *Vibrio bubulus*, representative strains of both species were tested against 10 antibiotics.

Minimum inhibiting levels of bacitracin, chloramphenicol, penicillin G, oxytetracycline, and tetracycline for *V. fetus* were higher, and those of dihydrostreptomycin and erythromycin lower, in thiol agar than in blood agar. Inhibiting levels of novobiocin, neomycin, and polymyxin B in thiol agar were similar to those in blood agar. In thiol medium, penicillin was relatively inactive, and polymyxin B was more active than in blood agar or thiol agar.

Vibrio bubulus was more resistant to novobiocin and dihydrostreptomycin and less resistant to bacitracin, penicillin, and polymyxin B in blood agar than was *V. fetus*. The difference in sensitivity of *V. bubulus* and *V. fetus* to novobiocin in blood agar appeared to be sufficient to aid in separating the 2 organisms.

INTRODUCTION

Bovine microaerophilic vibrios can usually be classified as *Vibrio fetus* (catalase positive, H_2S negative), a pathogen; or as *Vibrio bubulus* (catalase negative, H_2S positive), regarded as nonpathogenic. The latter commonly occurs in bull semen and must be differentiated from *V. fetus*.

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In vitro sensitivity trials have indicated that most *V. fetus* strains are resistant to bacitracin, are sensitive to chloramphenicol, erythromycin, dihydrostreptomycin, neomycin, and the tetracyclines, and may or may not be sensitive to penicillin and polymyxin B.[1,3,5,6] Variation between strains and the results of different workers may, in part, be due to differences in the medium used, the incubation period, and the gaseous environment. In the presence of liquid thiol medium, penicillin was found to be unstable.[4] Information on the antibiotic sensitivity of *V. bubulus* is not available.

For the purpose of determining the effect of the medium on the antibiotic sensitivity of *V. fetus* and comparing the antibiotic sensitivity of *V. fetus* and *V. bubulus*, representative strains of both species were tested against 10 antibiotics.

Materials and Methods

Cultures.—Of 19 *V. fetus* isolates used, 6 were obtained from bovine fetuses and 13 from bull semen. All of the 15 isolates of *V. bubulus* were obtained from bull semen.

Procedure.—Stock solutions of the antibiotics were prepared and added to the basal mediums to give the desired concentrations. The concentrations used were in multiples of 2.

The basal mediums were (1) blood agar (5.0% defibrinated ox blood in nutrient agar prepared with 0.3% beef extract [Difco]), 1.0% proteose peptone (Difco), and 0.5% NaCl, adjusted to pH 7.2 to 7.4; (2) thiol agar prepared by adding 2.0% of agar to thiol medium (Difco); and (3) thiol medium. The required amounts of the antibiotic solutions were added to the mediums after sterilization and cooling to 45 C. The solid mediums were dispensed in 12-ml. amounts in sterile petri dishes, allowed to solidify, stored at 10 C., and inoculated the following day. The thiol mediums were dispensed in 8 ml. amounts in ⅝ by 5-inch screw-capped test tubes, stored at 10 C., and inoculated the following day.

The inoculum was prepared by suspending cells from a 48-hour-old thiol agar slant culture in tryptose broth to yield a density corresponding to 1.0 on the McFarland nephelometer scale: 0.02 ml. was streaked on the surface of the agar plates, and 0.02 ml. was inoculated into the upper 3rd of the column of thiol medium.

The inoculated plates and tubes of antibiotic-thiol medium (screw-cap loose) were incubated

at 37 C. for 5 days in jars containing 5.0% oxygen, 10% carbon dioxide, and 85% nitrogen, the optimum gaseous environment for *V. fetus*.[2]

The minimum inhibiting level for a given antibiotic in the agar mediums was the lowest concentration that resulted in fewer than 20 visible colonies; in thiol medium it was the lowest concentration that prevented visible growth.

Influence of Medium.—The mode and range of minimum inhibiting levels of 10 antibiotics for *V. fetus*, based on the average of 2 to 4 trials, are summarized (Table 1).

agar. Chloramphenicol and the tetracyclines gave similar results in thiol medium and blood agar. Dihydrostreptomycin, erythromycin, and neomycin were more active in thiol medium than in blood agar. Of the antibiotics tested, penicillin G and polymyxin B were most obviously influenced by the culture medium. The modal inhibiting level of penicillin in blood agar was 2 units per milliliter and in thiol medium more than 160 units. In contrast, the modal inhibiting level of polymyxin B in blood agar was 320 units per milliliter and in thiol medium 20 units.

TABLE 1—Influence of Medium on the Sensitivity of Vibrio fetus

Antibiotic	Inhibiting level (units or μg./ml.) in Blood agar Mode	Blood agar Minimum	Blood agar Maximum	Thiol agar Mode	Thiol agar Minimum	Thiol agar Maximum	Thiol medium Mode	Thiol medium Minimum	Thiol medium Maximum
Bacitracin	160.0	40.0	320.0	640.0	160.0	640.0	320.0	160.0	640.0
Chloramphenicol	1.6	0.8	3.2	3.2	0.8	6.4	1.6	0.8	3.2
Dihydrostreptomycin	4.0	2.0	8.0	2.0	1.0	4.0	2.0	0.5	4.0
Erythromycin	3.2	0.8	6.4	1.6	0.8	3.2	0.4	0.1	0.8
Neomycin sulfate	8.0	4.0	16.0	8.0	4.0	16.0	4.0	1.0	4.0
Novobiocin*	32.0	16.0	64.0	32.0	16.0	64.0	16.0	8.0	128.0
Oxytetracycline	2.0	0.5	4.0	8.0	2.0	16.0	1.0	0.25	4.0
Penicillin G	2.0	0.125	16.0	8.0	0.5	16.0	>160.0	80.0	>160.0
Polymyxin B	320.0	5.0	640.0	320.0	5.0	320.0	20.0	2.5	40.0
Tetracycline	0.5	0.125	1.0	1.0	0.5	4.0	0.5	0.125	1.0

* Albamycin; the Upjohn Co., Kalamazoo, Mich.

Bacitracin, chloramphenicol, oxytetracycline, penicillin G, and tetracycline HCl were more active in blood agar than in thiol agar. In contrast, the ability of dihydrostreptomycin and erythromycin to inhibit growth of *V. fetus* was greater in thiol agar than in blood agar. The inhibiting levels of novobiocin sodium,* neomycin sulfate, and polymyxin B were similar for the 2 mediums. With both mediums, the range of inhibiting levels for individual strains was 3 or 4 dilutions for 8 of the antibiotics A wide range, 6 to 8 dilutions, was obtained with penicillin and polymyxin B in both blood agar and in thiol agar.

In liquid thiol medium, novobiocin appeared to be more active against some strains and less active against others than in the agar mediums. Bacitracin was less active in thiol medium than in blood

* Supplied as Albamycin by the Upjohn Co., Kalamazoo, Mich.

Sensitivity of V. bubulus.—The *V. bubulus* cultures were tested against the 10 antibiotics in blood agar. The minimum inhibiting levels of chloramphenicol, erythromycin, neomycin, oxytetracycline, and tetracycline were similar (within limits of 2 dilutions) to those obtained with *V. fetus*. The 2 organisms differed in sensitivity to 5 antibiotics (Table 2).

Based on the modal levels, *V. bubulus* was about 8 times as resistant to novobiocin in blood agar as was *V. fetus*. With the strains examined, the difference was sufficient to provide an additional means of separating the 2 organisms. At the 64 units/ml. level in blood agar, all *V. bubulus* cultures and none of the *V. fetus* cultures produced visible colonies. However, differences in sensitivity to novobiocin in thiol agar and thiol medium were not sufficient to separate the 2 organisms.

V. bubulus was more sensitive to bacitracin, penicillin G and polymyxin B, and less sensitive to dihydrostreptomycin, than was *V. fetus*.

TABLE 2—Sensitivity of Vibrio bubulus and Vibrio fetus to Several Antibiotics

Antibiotic	Inhibiting level (units or μg./ml.)* *V. bubulus* Mode	*V. bubulus* Minimum	*V. bubulus* Maximum	*V. fetus* Mode	*V. fetus* Minimum	*V. fetus* Maximum
Bacitracin	40.0	40.0	80.0	160.0	40.0	320.0
Dihydrostreptomycin	16.0	4.0	16.0	4.0	2.0	8.0
Novobiocin	256.0	128.0	256.0	32.0	16.0	64.0
Penicillin G	0.125	0.125	1.0	2.0	0.125	16.0
Polymyxin B	10.0	2.5	20.0	320.0	5.0	640.0

* In blood agar.

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SUMMARIO IN INTERLINGUA

Sensibilitate pro Antibioticos in Vibriones Bovin

Pro determinar le effecto del medio super le sensibilitate de *Vibrio fetus* pro antibioticos e pro comparar le sensibilitate pro antibioticos inter *V. fetus* e *V. bubulus*, racias representative del duo species esseva testate contra 10 antibioticos.

Le nivellos minime de inhibition de *V. fetus* in le caso de bacitracina, chloramphenicol, penicillina G, oxytetracyclina e tetracyclina esseva plus alte, illos in le caso de dihydrostreptomycina e erythromycina esseva plus basse in agar a thiol que in agar a sanguine. Le nivellos inhibitori de novobiocina, neomycina, e polymyxina B in agar a thiol esseva simile a illos in agar a sanguine. In le medio a thiol, penicillina esseva relativemente inactive, sed polymyxina B esseva plus active que in agar a sanguine o in agar a thiol.

V. bubulus in agar a sanguine esseva plus resistente que *V. fetus* contra novobiocina e dihydrostreptomycina e minus resistente contra bacitracina, penicillina, e polymyxina B. Le differentias de sensibilitate inter *V. bubulus* e *V. fetus* pro novobiocina in agar a sanguine pareva esser sufficiente pro esser de adjuta in separar le 2 organismos.

Antibiotic Mediums for the Isolation of Vibrios from Bull Semen

W. N. Plastridge, Ph.D.; M. E. Koths, B.S.; L. F. Williams, M.S.

SUMMARY

Of 231 semen samples, 19 were positive for *Vibrio fetus* when cultured on blood agar, 26 when cultured on blood agar containing 2.0 units of bacitracin per milliliter, and 33 were positive when cultured on blood agar containing 2 μg. of novobiocin per milliliter.

Of 285 samples cultured on blood agar and on blood agar plus 2 units/ml. of bacitracin and 2 μg./ml. of novobiocin, 13 were *V. fetus*-positive on blood agar and 31 were *V. fetus*-positive on the antibiotic medium.

The average number of *Vibrio*-like colonies obtained from semen samples was reduced and the percentage of such colonies found to be vibrios was greatly increased by the use of blood agar containing bacitracin and novobiocin, separately and in combination. Bacitracin alone was less effective than novobiocin in preventing crowding of plates with large colony-forming bacteria.

Bacitracin-novobiocin blood agar medium was definitely superior to blood agar in culturing semen for the detection of *V. fetus*.

The need for separating *V. fetus* from *V. bubulus* was emphasized.

INTRODUCTION

Identification of *Vibrio fetus*-carrier bulls requires isolation of the organism from semen or preputial washings, both of which usually contain other microorganisms and, frequently, catalase negative (nonpathogenic) vibrios. Semisolid mediums have been used for isolating vibrios.[2-4,7] To suppress the growth of organisms other than vibrios, Schneider and Morse[7] added ox bile (10%) to Albimi brucella broth with 0.1% agar; Mundt[4] used different levels of streptomycin in thiol medium; and Kuzdas and Morse[3] added ox bile (4%), ethyl violet (1:800,000), bacitracin (25 units/ml.), polymyxin B (5 units/ml.), and cycloheximide* (0.1 mg./ml.) to Albimi brucella broth containing 0.5% agar. Owing to the need for differentiating between *V. fetus* and other vibrios, semisolid mediums are not satisfactory for detecting *V. fetus*-carrier bulls. Blood agar plates, inoculated with bull semen and incubated in jars containing the optimum gaseous environment for *V. fetus*, were used by Plastridge *et al.*[6] This permitted the subculturing and identification of *Vibrio*-like colonies. However, the presence of other organisms in large numbers and non-*Vibrio* organisms that produced *Vibrio*-like colonies impaired the efficacy of the test.

The relatively high level of tolerance of bovine vibrios for novobiocin** and bacitracin, reported by Plastridge and Koths,[5] suggested the use of these agents in blood agar for culturing bull semen. The results obtained are reported.

Materials and Methods

Semen samples, in 0.5- to 1.0-ml. amounts, were obtained from 145 bulls at 3 New England artificial insemination centers. The samples were packed in insulated containers with a can of ice and delivered to the laboratory within 24 hours following collection.

The mediums used were (1) blood agar prepared by adding 5.0% defibrinated ox blood to an agar

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* Actidione, produced by the Upjohn Company, Kalamazoo, Mich.

** Novobiocin sodium (Albamycin) and bacitracin were obtained from the Upjohn Company, Kalamazoo, Mich.

TABLE 1—Semen Cultured on Blood Agar With and Without Added Antibiotics

No. of samples	Medium	No. of samples				
		Negative	Unsatisfactory*	*V. bubulus*	*V. fetus* and *V. bubulus*	*V. fetus*
231	Blood agar	141	58	13	1	18
	Bacitracin**	102	63	40	7	19
	Novobiocin†	113	23	63	9	24
285	Blood agar	209	52	12	1	12
	Bacitracin and novobiocin‡	142	21	90	9	22

* Plates were overgrown with "saprophytic" bacteria. ** 2 units/ml. † 2 μg./ml. ‡ Bacitracin, 2 units/ml., and novobiocin, 2 μg./ml.

base (45 C.) consisting of 0.3% beef extract (Difco), 1.0% proteose peptone (Difco), 0.5% sodium chloride, and adjusted to pH 7.2–7.4, (2) blood agar plus 2 units per ml. of bacitracin, (3) blood agar plus 2 micrograms per ml. of novobiocin, (4) blood agar with both antibiotics, and (5) blood agar with both antibiotics plus 1.0% dehydrated ox bile (Difco). The plates were incubated overnight at 37 C. to remove excessive moisture, and were either used on the day after preparation or after storage at 5 C. for periods up to 6 days.

The stock antibiotic solutions were prepared by dissolving 100,000 units of bacitracin in 50 ml. of sterile water (2,000 units/ml.) and 500 mg. of novobiocin in 100 ml. of water (5,000 μg./ml.); they were stored at −10 C. up to 30 days. Routinely, 0.25 ml. of the bacitracin solution and 0.1 ml. of the novobiocin solution were added to 250 ml. of the agar base, at the same time as was the defibrinated blood.

Two plates were inoculated with each sample; one, by streaking 0.01 ml. of semen plus a loopful of thiol medium (Difco) over the surface, and the other by streaking the surface with 0.02 ml. of a 1:100 dilution of semen in thiol medium.

After incubation for 3 days in jars containing 5% oxygen, 10% carbon dioxide, and 85% nitrogen, the plates were examined with the aid of a 3x lens (5-inch diameter), using reflected light. Small, translucent, light-gray colonies (0.3- to 1.0-mm. diameter) were transferred to tubes of thiol medium (8 ml.). Up to 10 colonies per plate were subcultured. Subcultures containing *Vibrio*-like growth were examined under a phase-contrast microscope. If vibrios were seen, a blood agar plate was inoculated and incubated aerobically; a tube of rehydrated triple sugar iron agar (Difco) was inoculated, using a loop and the stab method; and 5.0 ml. of the culture was mixed with 5.0 ml. of a 3% solution of hydrogen peroxide in a Smith fermentation tube. Microaerophilic vibrios that were catalase positive (producing over 5.0 mm. of oxygen) and H_2S negative (no blackening of the TSI medium in 48 hours) were classed as *V. fetus*, and those that were catalase negative and H_2S positive were classed as *Vibrio bubulus*.

Results

Semen "Contaminants" Inhibited by Bacitracin and Novobiocin.—Isolations from bull semen that were inhibited by 2.0 μg./ml. of novobiocin in blood agar were: 3 nonhemolytic diphtheroids, 2 of 4 nonhemolytic *Streptococcus* cultures, a *Staphylococcus*, *Bacillus subtilis*, and an unidentified gram-negative rod. Bacitracin at the 2.0 unit/ml. level inhibited one *Streptococcus* culture that was not inhibited by novobiocin and failed to suppress the growth of *B. subtilis*. Neither agent pre-

TABLE 2—Vibrio-like Colonies Resulting from Culture of Semen Samples on Blood Agar and Selective Mediums

Vibrio fetus	Medium	No. of samples*	Isolations		Percent of colonies	
			Number	Average	*V. bubulus*	*V. fetus*
Positive (1 or more mediums)	Blood agar	67	411	6.1	1.4	16.2
	Bacitracin**	38	173	4.6	6.9	52.6
	Novobiocin†	40	203	5.1	11.8	43.8
	Bacitracin and Novobiocin‡	32	147	4.6	9.5	66.0
Negative	Blood agar	375	1,634	4.4	2.2	
	Bacitracin**	142	283	2.0	25.8	
	Novobiocin†	176	539	3.1	24.7	
	Bacitracin and Novobiocin‡	232	592	2.6	38.7	

* Contaminated samples omitted. ** 2 units/ml. † 2 μg./ml. ‡ Bacitracin, 2 units/ml., and novobiocin, 2 μg./ml.

vented growth of *Pseudomonas aeruginosa* and *Proteus vulgaris*, although the tendency for "spreading" by *P. vulgaris* appeared to be reduced by novobiocin.

Blood Agar With and Without Antibiotics.—A total of 231 semen samples was cultured on blood agar, bacitracin (2 units/ml.) blood agar, and novobiocin (2μg./ml.) blood agar; 285 were cultured on blood agar and bacitracin-novobiocin blood agar (Table 1).

Less interference from large colony-forming "contaminants" occurred when novobiocin alone, or in combination with bacitracin, was added to the blood agar. Bacitracin alone was not effective for this purpose.

The number of samples that yielded vibrios was markedly increased by culturing on blood agar containing either or both antibiotics. The increase occurred for both *V. bubulus* and *V. fetus*.

Of the 231 samples, 40 yielded *V. fetus* alone or in combination with *V. bubulus* on 1 or more of the 3 mediums. The number of samples that were *V. fetus*-positive on blood agar was 19; on bacitracin blood agar, 26; and on novobiocin blood agar, 33. Of the 7 samples that were negative on the novobiocin medium, 2 were positive on blood agar and 5 on the bacitracin medium.

Blood agar was compared with blood agar containing bacitracin and novobiocin in tests made on 285 samples. The numbers of samples that were *V. fetus*-positive on the 2 mediums were 13 and 31, respectively. One sample only was positive on blood agar and negative on the antibiotic medium.

The addition of 1.0% dehydrated ox bile (Difco) to the antibiotic blood agar medium suppressed the growth of both *V. fetus* and *V. bubulus*. When 48 additional semen samples were cultured on bacitracin-novobiocin blood agar, 17 yielded *V. bubulus;* 4, *V. bubulus* and *V. fetus;* and 4, *V. fetus*. On the ox bile medium, no visible *Vibrio* colonies were obtained.

Colonies Identified as Vibrio.—The antibiotics suppressed the development of *Vibrio*-like colonies of organisms other than *Vibrio*, as shown by a reduction in the average number of isolations and a higher percentage of colonies isolated and found to be vibrios (Table 2).

With *V. fetus*-positive samples, the percentage of *Vibrio*-like colonies identified as *V. fetus* was 16.2% for blood agar, 52.6% for the bacitracin medium, 43.8% for the novobiocin medium, and 66.0% for bacitracin-novobiocin blood agar. The percentages identified as *V. bubulus* were 1.4, 6.9, 11.8, and 9.5%, respectively.

The percentage of *Vibrio*-like colonies from the *V. fetus*-negative samples identified as *V. bubulus* was 2.2% for blood agar, 25.8% for bacitracin blood agar, 24.7% for novobiocin blood agar, and 38.7% for bacitracin-novobiocin blood agar.

These results emphasize the need for (1) isolating all *Vibrio*-like colonies, or at least 10 colonies if large numbers are present, and (2) identifying the vibrios isolated in the detection of *V. fetus*-carrier bulls.

For the routine differentiation of *V. fetus* from other vibrios found in bull semen, the catalase and H_2S tests, as suggested by Bryner and Frank,[1] appear to be adequate if the vibrios are definitely microaerophilic (fail to grow, or produce a few colonies only, when inoculated onto blood agar and incubated aerobically). In the present investigation, 4 semen samples yielded cultures which grew abundantly on blood agar after 24 hours of incubation in air. All 4 failed to grow in 3.5% NaCl in thiol medium. Two were catalase positive and H_2S negative, and were tentatively identified as *Vibrio percolans*. One was catalase positive and H_2S positive, and one was catalase negative and H_2S negative. The aerobic vibrios are regarded as nonpathogenic.

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SUMMARIO IN INTERLINGUA

Medios Antibiotic pro le Isolation de Vibriones ab le Semine de Tauros

Inter 231 specimens de semine, 19 esseva positive pro *Vibrio fetus* in culturas super agar a sanguine continente bacitracina in un concentration de 2,0 unitates per millilitro, e 33 esseva positive in culturas super agar a sanguine continente novobiocina in un concentration de 2 μg per millilitro.

Inter 285 specimens in culturas super agar a sanguine e super agar a sanguine continente bacitracina in un concentration de 2 unitates per millilitro e novobiocina in un concentration de 2 μg per millilitro, 13 esseva positive pro *V. fetus* super agar a sanguine sin antibioticos e 31 super agar a sanguine con antibioticos.

Le numero medie de colonias resimilante *Vibrio* que esseva obtenite ab le specimens de semine esseva reducite e le procentage de tal colonias identificate como realmente colonias de *Vibrio* esseva grandemente augmentate per le uso de agar a sanguine continente bacitracina e novobiocina—separatemente e in combination. Bacitracina sol esseva minus efficace que novobiocina in prevenir le congestion del placas con bacterios formante grande colonias.

Agar sanguinee a bacitracina-novobiocina se monstrava como un medio definitemente superior a agar sanguinee in le culturation de semine pro le detection de *V. fetus*.

Le necessitate de separar *V. fetus* ab *V. bubulus* es sublineate.

Studies in Anaplasmosis. III. An Autoantibody and Symptomatic Macrocytic Anemia

Miodrag Ristic, D.V.M., Ph.D.

SUMMARY

In this study, an autoantibody directed against the erythrocytes of animals infected with Anaplasma marginale was demonstrated.

That the anemia of anaplasmosis is a symptomatic macrocytic one was ascertained by observing compensatory bone marrow hyperactivity through its manifestations in the peripheral blood. These manifestations included granulocytosis, reticulocytosis, increased mean corpuscular cell volume, and increased osmotic fragility of the erythrocytes.

The coincidence of the maximum anemia, as revealed by hematocrit value, and the maximum concentration of autoantibody attached to the erythrocytes suggested a causal relationship of these 2 processes in bringing about symptomatic macrocytic anemia associated with anaplasmosis.

Supporting data concerning the mechanism by which the autoantibody may contribute to the development of anemia in anaplasmosis suggest that the erythrocytes sensitized with autoantibody are removed from the circulation and broken down in cells of the reticulo-endothelial system. There was no indication that the activity of autoantibody might result in intravascular hemolysis.

INTRODUCTION

While autoimmunization is known to occur in man,[3, 5, 6] there has been no report of autoantibody formation under natural conditions in domestic animals. In this work, consideration is given to the existence of such an antibody directed against the erythrocytes of animals infected with *Anaplasma marginale*. In addition, a causal relationship between the autoantibody and the presence of a symptomatic macrocytic anemia is elucidated.

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From the Department of Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana, and the Department of Veterinary Science, University of Florida, Gainesville.

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The author is indebted to Mr. Ray Creel for technical assistance.

Materials and Methods

Two Guernsey bull calves (No. 45 and 50), approximately 6 months old and infected with 50 ml. of carrier blood each, were used to study the occurrence of the autoantibody and concomitant hematologic abnormalities. In addition, an 18-month-old Guernsey heifer was used to ascertain the effect of splenectomy on the course of the autoantibody reaction. This animal was infected with 20 ml. of carrier blood and splenectomized approximately 2 months later, at the time the first hematologic signs of convalescence were observed.

Detection of the Autoantibody Occurring on the Erythrocytes.—Blood samples from cattle infected with *A. marginale* were drawn from the jugular vein into an equal volume of Alsever's solution. The erythrocytes were washed 3 times in normal physiologic saline solution. The autoantibody fixed on the erythrocytes was detected in a hemolytic system which was set by adding 2 drops of rabbit complement to 2 drops (approximately 0.05 ml. per drop) of a 2% suspension of erythrocytes in physiologic saline solution. The degree of lysis was determined after the system had been incubated for 4 hours at 20 to 24 C. Control tests were also made, using erythrocytes of infected animals plus saline solution alone, erythrocytes of infected animals plus heat-inactivated complement, and erythrocytes of a normal animal plus rabbit complement. Fresh, undiluted rabbit serum proved to be the most satisfactory source of complement for the test.

Specific Absorption of Rabbit Complement.—The purpose of this test was to demonstrate that the active component in the rabbit serum was the complement which was capable of reacting with the specific antibody-antigen system, and not a naturally occurring hemolysin. This process involved removing the complement with a precipitin complex consisting of egg albumin-anti-egg albumin antibody. The optimal ratio of antigen to antibody was estimated by determining the quantity of precipitate formed using various concentrations of these components. The system employed for absorption of complement consisted of 1 ml. of 1:10

egg albumin in physiologic saline solution plus an equal volume of undiluted specific rabbit serum. These were admixed and incubated in a water bath at 37 C. for 1 hour. The resulting precipitate was washed 3 times in physiologic saline solution admixed with 1 ml. of fresh rabbit serum, and incubated for an additional 30 minutes. The precipitate-complement complex was removed by centrifugation, and the supernatant fluid was used in the test for detection of autoantibody as described. Adequate control rabbit serum, treated in a similar manner, without specific precipitate, was employed.

Dissociation of Autoantibody from Sensitized Erythrocytes and Its Adsorption on Normal Erythrocytes.—The stromas of erythrocytes were obtained by the method of Komninos and Rosenthal,[9] and the antibody elution technique used was that of Landsteiner and van der Scheer.[10]

The source of erythrocytes, sensitized *in vivo* with the autoantibody, was approximately 800 ml. of citrated infected bovine blood. The autohemolysis titer of the erythrocytes was 4+. From this blood 200 ml. of packed erythrocytes was collected. The cells were washed 5 times with physiologic saline solution. The cells were then firmly packed by centrifugation and hemolyzed with 10 times their volume of distilled water which had been saturated with CO_2 at 4 C. The precipitated stromas were washed repeatedly with distilled water, buffered at pH 5.6 to 5.8, and alternately frozen and thawed after every third washing. The stromas were considered to be satisfactory for the elution of autoantibody when they appeared off-white in color. The stromas were then suspended in twice their volume of physiologic saline solution, thoroughly mixed, and incubated at 56 C. for 15 minutes. Following this, the preparation was centrifuged for 30 minutes in prewarmed cups at 5,000 r.p.m. in a centrifuge with a 12-inch diameter head. The resulting supernatant fluid containing the eluted autoantibody was used to sensitize the erythrocytes obtained from a normal animal by admixing approximately 1 part of the cells with 10 parts of the supernatant fluid. After incubation of the mixture for 1 hour at 37 C., the cells were washed 3 times by centrifugation, after which the autoantibody test was performed as described. A control test consisted of normal erythrocytes treated with saline solution instead of saline solution containing eluted autoantibody.

Hematologic Studies.—Heparinized blood samples (0.5 mg. of heparin sodium/1.0 ml. of blood) were used throughout the entire study. The procedures outlined by Gradwohl[7] were employed for leukocyte and erythrocyte counts and for determination of hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, and packed cell volume. For enumeration of reticulocytes, the method of Cook-Meyer-Turner[7] was used. Osmotic fragility of erythrocytes was ascertained according to the method of Sanford.[16] Blood films stained with Giemsa's stain were used to observe and enumerate the marginal *Anaplasma* bodies.

Results

The results of autoantibody activity on erythrocytes and the blood findings of calf 45 are shown (Fig. 1, 2). The maximum autoantibody response closely coincided with the lowest hematocrit value. In all the animals studied, the maximum proportion of infected erythrocytes and the highest complement-fixing titer preceded the 2 reactions. The interval between the peak of infection and the maximum autoantibody response varied between 2 and 8 days. During this interval, a constant decrease of the hematocrit value was seen, and the lowest values were recorded usually at the end of the period. This period was immediately followed by gradual increase of the erythrocyte count. Hematologic studies revealed marked granulocytosis preceded by moderate reticulocytosis. The highest concentration of reticulocytes, 4.5%, occurred at the time the packed cell volume was the lowest. The maximum granulocytosis, 21,000/cmm., was demonstrated shortly after the first improvement in the hematocrit value. Osmotic fragility was manifested by an increase in beginning lysis of erythrocytes from 0.62% NaCl, at the beginning of infection to 0.73% NaCl at the time the first marginal *Anaplasma* bodies were demonstrated. An increased mean corpuscular volume of approximately 2.5 times normal was noticed at the time the hemoglobin and hematocrit values had a definite increase. Although at fluctuating levels, the macrocytes persisted during the period of convalescence.

The sequence of immunologic and hematologic reactions of calf 50 were similar to those of calf 45. Calf 50, however, had the maximum number of parasitized erythrocytes at 21 days following infection and demonstrated the highest autoantibody titer of a 3+ reaction.

At the time of splenectomy, the 18-month-old heifer had a complement-fixing titer of 1:40, a packed cell volume of 27%, a 2% infection rate of its erythrocytes and an autohemolysis titer of 2+ (Fig. 3). During the 7 days following splenectomy, there was a very slight fluctuation of the hematocrit value and the autoantibody level. By the end of the 7-day period, the complement-fixing titer receded to 1:20 and a packed cell volume of 19% was recorded. Forty-eight hours later, demonstrable autoantibody disappeared, hemato-

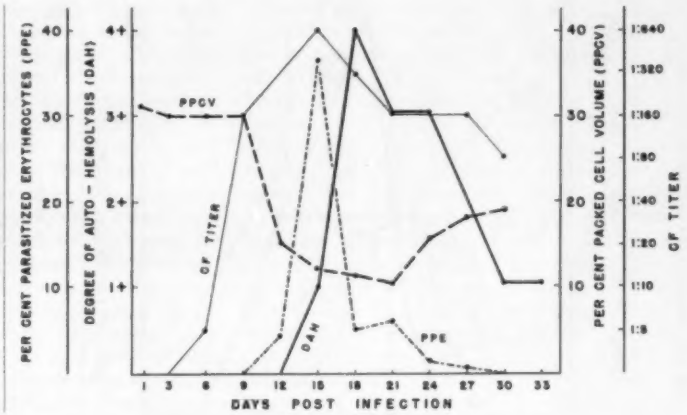

Fig. 1—Autoantibody activity on erythrocytes of a 6-month-old Guernsey calf infected with 50 ml. of blood from an anaplasmosis carrier.

crit level decreased to 13%, and as much as 20% of the erythrocytes contained marginal *Anaplasma* bodies. Fourteen days following splenectomy, the animal had the following blood picture: hematocrit value 11%, complement-fixing titer 1:10, 40% of the erythrocytes containing marginal bodies, and no demonstrable autoantibody. Two days later the animal died.

Fresh rabbit serum which was used in the albumin-anti-egg-albumin antibody system had no complement activity when subsequently used in the autohemolytic system.

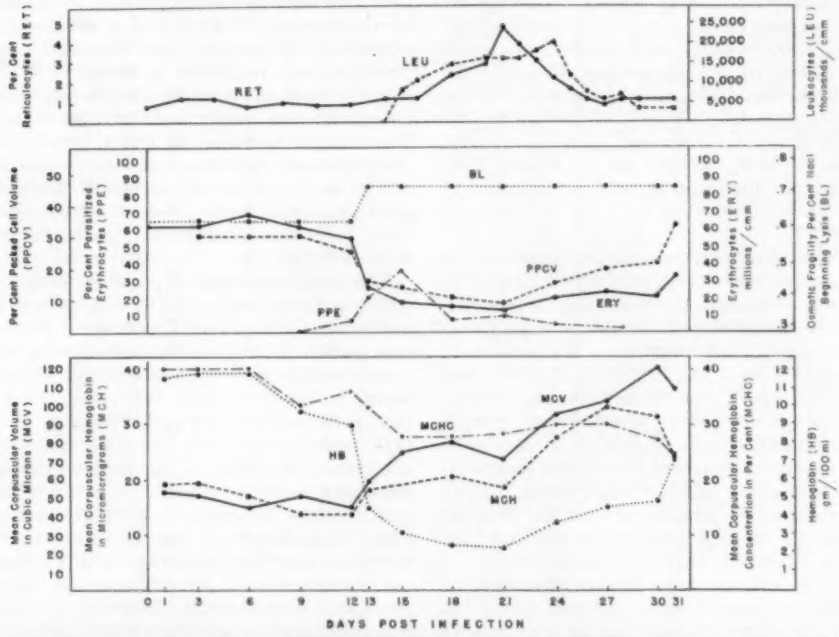

Fig. 2—Blood pathology of a 6-month-old Guernsey calf infected with 50 ml. of blood from an anaplasmosis carrier.

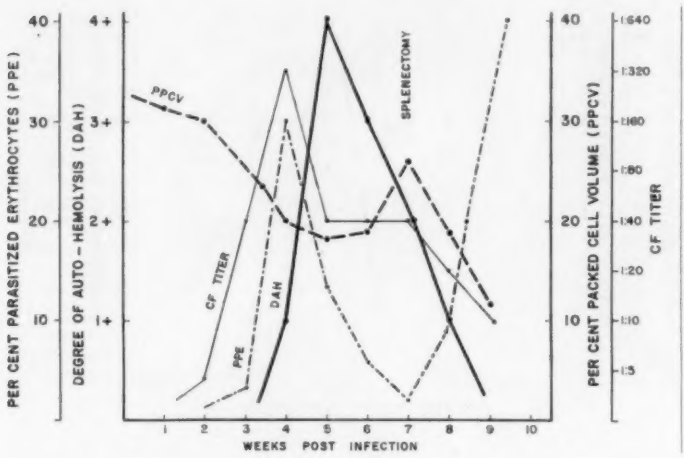

Fig. 3—The effect of splenectomy on the cause of autoantibody activity in an 18-month-old Guernsey heifer infected with 20 ml. of blood from an anaplasmosis carrier.

When normal erythrocytes sensitized with eluate from the autoantibody-containing erythrocytes were exposed to rabbit complement, lysis occurred at a 2+ reading. No lysis occurred in the control where physiologic saline solution and rabbit complement acted upon erythrocytes.

Determination of thermal requirements for the reaction of the autoantibodies, the "cold" or "warm" agglutinins, was made by setting paired specimens of sensitized erythrocytes and rabbit complement at 24 C. and at 4 C. Three to four hours were required for the maximal lysis of erythrocytes at 24 C., and 8 to 12 hours were needed for similar reaction to occur at 4 C.

Discussion

While the exact mechanism involved in the production of autoantibody in anaplasmosis is unknown, the following hypotheses have been advanced as possible explanations of this phenomenon. According to Wiener,[18] bacteria, viruses, toxins, and other hemagglutinating or hemolytic agents may alter erythrocytes and thus render them autoantigenic. He drew attention to the similarity between these reactions and the augmenting effect of adjuvants. Najjar and Robinson [11] remarked that the damage caused to the cell surface by the reaction of the attached antibody would result in autolysis and a breakdown or alteration of the tissue component to exposed antigenic sites. In this manner, the production of autoantibody may become self-perpetuating. Contrary to Wiener's and Najjar and Robinson's reasoning, Kissmeyer-Nielsen *et al.*[8] postulated that the true etiology of autoimmunization is unknown and that it occurs as a consequence of increased blood destruction.

Regardless of the exact *modus operandi* of *A. marginale*, the simultaneous appearance of a high degree of anemia, as revealed by the hematocrit level and a marked concentration of autoantibody attached to the erythrocytes, indicates a causal relationship between the concentration of autoantibody and the occurrence of the anemia. The drastic increase in mean corpuscular cell volume (appearance of macrocytes), in addition to increased osmotic fragility, granulocytosis, and moderate reticulocytosis, indicates compensatory bone marrow hyperactivity.

There are at least 3 possible means by which the occurrence of autoantibody could contribute to increased erythrocyte destruction and thus lead to the appearance of a symptomatic macrocytic anemia in *A. marginale*-infected animals. (1) Hemolysis of the erythrocytes could result from action of free complement and from the increased mechanical fragility of the antibody-coated cells, particularly if the cells should adhere to each other and become suddenly and violently separated in the circulation. (2) Intravascular agglutination with mechanical trauma acting on erythrocytic aggregates, and sequestration followed by hemolysis of agglutinated cells in the spleen and other organs could take place. According to Castle *et al.*,[2] the agglutination also

brings about hemolysis via erythrostasis with local exclusion of serum, tissue ischemia, and the release of injurious substances from autolyzed tissues adjacent to stagnating red cells. In this connection, attention is called to the frequent occurrence of petechial hemorrhages on the pleura and diaphragm, and on the pericardium of animals which have succumbed to anaplasmosis.[4,13] According to Wasastjerna,[17] Faohraeus stated that lysolecithin may be responsible to some degree for the destruction of clumped erythrocytes which remain for longer periods than normal cells in sequestering organs, such as the spleen. (3) The autoantibody may act as a hemopsonin, thus rendering the erythrocytes susceptible to phagocytosis. Phagocytes, predominantly large monocytes containing ingested erythrocytes, have been observed at certain stages of acute anaplasmosis.

In considering these mechanisms by which the autoantibody could contribute to the elimination of circulating erythrocytes, the hemolytic phenomenon in anaplasmosis does not appear probable because of *(a)* extremely low content of free complement in the bovine serum; *(b)* absence of any considerable increase of free hemoglobin in the plasma during the period of progressive anemia in experimentally infected animals; and *(c)* absence of *in vitro* lysis of sensitized erythrocytes in the presence of fresh normal bovine serum. Baker and Douglas' studies[1] with Fe^{59} also tend to preclude intravascular hemolysis and support the hypothesis that hemoglobin is broken down in cells of the reticulo-endothelial system, primarily those of the spleen.

The concentration of autoantibody on the erythrocytes was apparently unrelated to the number of erythrocytes with marginal *Anaplasma* bodies. This fact indicates that there is little immunogenic relationship between the *Anaplasma* body and the autoantibody described here. However, only partial lysis of normal erythrocytes resulted following their exposure to the dissociated autoantibody in the presence of complement. It is, therefore, possible that *(a)* this antibody is directed against the submicroscopic initial bodies which were observed in nearly every erythrocyte of an acutely infected animal;[12] *(b)* the autoantibody is directed against the matrixes of the initial and marginal bodies, this substance being a product of the cell-parasite interaction;[14] and *(c)* the autoantibody is directed against unmasked antigenic sites of erythrocytes, the latter being the result of the enzymatic activity of the antigen as in the Thomsen-Friedenreich phenomenon.[15] Under the circumstances, the mechanism of autoantibody production could be a consequence of an altered antigen, produced or exposed by integration of *Anaplasma* in the susceptible erythrocytes. This antibody, however, would possess the ability of cross-reacting with the unaltered native tissue molecule. Consequently, this may explain partial lysis of the erythrocytes of *Anaplasma*-free animals in the presence of an autoantibody-complement system.

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SUMMARIO IN INTERLINGUA

Studios in Anaplasmosis. III. Un Autoanticorpore e Symptomatic Anemia Macrocytic

In le presente studio, un autoanticorpore de activitate anti le erythrocytos de animales inficite con *Anaplasma marginale* esseva demonstrate.

Que le anemia de anaplasmosis es un symptomatic anemia macrocytic esseva provate per le observation de un hyperactivitate compensatori del medulla ossee, manifeste per alterationes in le sanguine peripheric. Iste alterationes includeva granulocytosis, reticulocytosis, augmento del volumine medie de cellulas corpuscular, e augmento del fragilitate osmotic de erythrocytos.

Le coincidentia del maximo de anemia (secundo le valores de hematocrite) e del maximo de concentration del autoanticorpore attachate al erythrocytos suggereva un relation causal de iste 2 processos in le causation del symptomatic anemia macrocytic que es associate con anaplasmosis.

Quanto al mechanismo per que le autoanticorpore contribue possibilemente al disveloppamento del anemia de anaplasmosis, certe datos corroboratori suggere que le erythrocytos sensibilisate con autoanticorpore es eliminate ab le circulation e decomponite in cellulas del systema reticulo-endothelial. Esseva trovate nulle indication que le activitate de autoanticorpore resulta possibilemente in hemolyse intravascular.

Electron Microscopy of *Anaplasma marginale* in the Bovine Erythrocyte

Walter L. Scott, B.S., M.S.; Jack C. Geer, M.D.; Lon E. Foote, D.V.M.

SUMMARY

Study of the *Anaplasma* body with the electron microscope has shown the body to be composed of a limiting membrane enclosing smaller units that appeared in 3 morphologic forms. The first 2 forms differed in the presence or absence of a central dense mass. The 3rd form, characterized by numerous dense particles in the small units within the *Anaplasma* body, was interpreted as artifactual distortion of the body. The results of this study were discussed in light of previous reports concerning the appearance of the *Anaplasma* body in the electron microscope.

This study indicates the *Anaplasma* body to be neither protozoan nor viral, but probably rickettsial.

INTRODUCTION

It is generally agreed that the etiologic agent of anaplasmosis resides in the *Anaplasma* body. Classification of the *Anaplasma* body is uncertain; it has been characterized as a protozoan,[10] a rickettsia,[3] and it has been suggested that it may be a virus.[9] Findings from electron microscopy have varied both in observed structure and interpretation. This investigation into the morphology of the *Anaplasma* body was made in order to assist in classification of the agent.

Materials and Methods

A splenectomized calf was inoculated intravenously with 10 cc. of blood from a carrier animal. After 15 days, 89% of the circulating red blood cells contained *Anaplasma* bodies on a Giemsa-stained smear. Blood was withdrawn from the external jugular vein and fixed for 30 minutes in cold 1% buffered osmium tetroxide[6, 12] with added sucrose.[6] Vestopal-W[11, 14] or a 1:3 mixture of methyl and butyl methacrylates was used for embedding. The blood was dehydrated in methyl alcohol and acetone for vestopal-W embedding, and in graded methyl alcohols for methacrylate embedding. Methacrylate was polymerized in an oven at 38 C. for 46 hours following the addition of benzoyl peroxide as a catalyst. Vestopal-W was hardened at 60 C. for 19 hours. Sections were cut on an ultra-microtome and mounted on formvar-coated grids. Lead hydroxide stain was applied to some methacrylate sections.[15]

Results

Sectioned erythrocytes containing *Anaplasma* bodies were readily found. The *Anaplasma* bodies, located for the most part at the margin of the erythrocyte, consisted of a space containing from 1 to 7 small units (Fig. 1). The space was limited by a 70 Å membrane (Fig. 2). The small units varied from 0.25 to 0.58 μ in diameter; their structure varied with the embedding medium. In methacrylate-embedded blood, the small units appeared separate and distinct from each other and from the membrane limiting the containing space (Fig. 4). In vestopal-embedded blood, the small units also were clearly separate from each other and the membrane limiting the larger space, but were closer to each other and to the space's limiting membrane (Fig. 1–3). The small units could be classified by structure into 1 of 3 general forms. The most common form, found both in vestopal-W and methacrylate-embedded blood, was composed of a central dense mass from which dense strands radiated in spoke-like fashion toward a limiting membrane (Fig. 3). Occasionally, the radiating strands appeared fused or ruptured (Fig. 1, 3). The limiting membrane of the units was composed of 2 smaller membranes, each 70 Å in thickness, separated by a 130-Å space (Fig. 1, 2).

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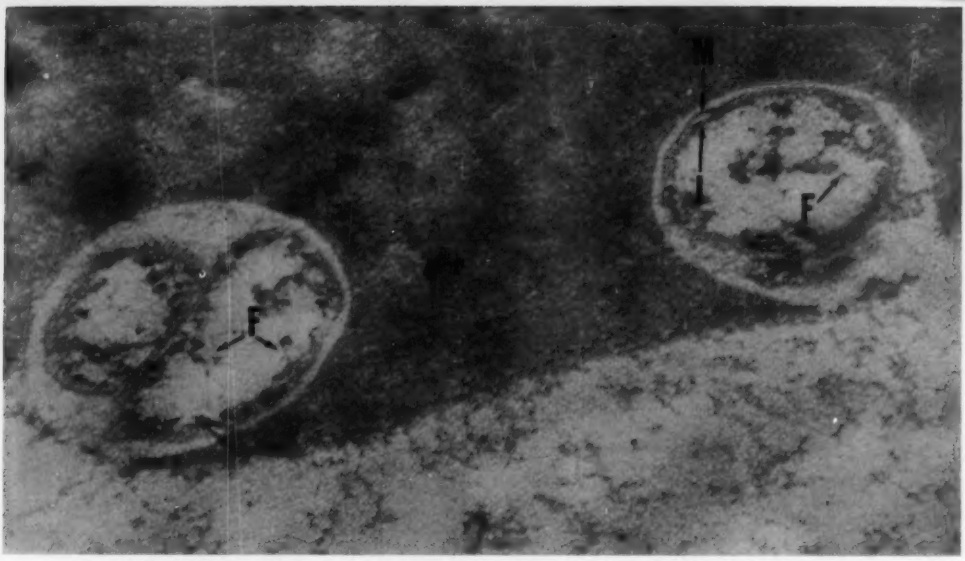

Fig. 1—Vestopal-W-embedded erythrocyte (Er) containing 2 Anaplasma bodies. On the right is an Anaplasma body containing a single small unit. On the left is an Anaplasma body containing 2 small units. The inner (I) and outer (M) layers of the limiting membrane are indicated. Notice strands and irregular masses of dense material within the small units (F). x 75,000

The 2nd form of the small unit, found both in vestopal-W and methacrylate-embedded blood, lacked the central dense area and contained strands and irregular masses of dense material, between which were areas of lesser density (Fig. 1, 4). The limiting membrane was similar to that of the 1st form.

The 3rd form of the small unit, found most often in methacrylate-embedded blood, was similar to the previous forms, except that the strands and irregular masses were largely replaced by dense particles approximately 100 Å in diameter. The dense particles were adjacent to the inner limiting membrane (Fig. 4) and the dense central mass when this was present.

Discussion

There is considerable confusion in the literature about the fine structure of the *Anaplasma* body. DeRobertis and Epstein,[7] using a replica technique with hemolyzed cells, observed the marginal *Anaplasma* body to consist of a central, undivided mass and peripheral elementary bodies of high electron density (170 to 220 mμ

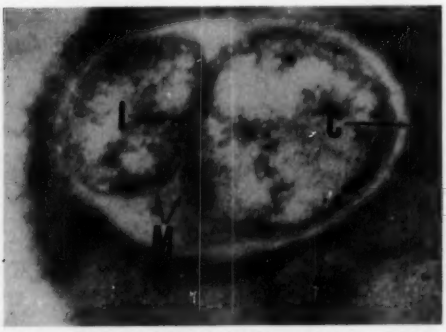

Fig. 2—Vestopal-W-embedded Anaplasma body containing 2 small units. Notice the inner (I) and outer (M) layers of the double membrane of the small units and the membrane of the containing space (C). Erythrocyte (Er). x 75,000

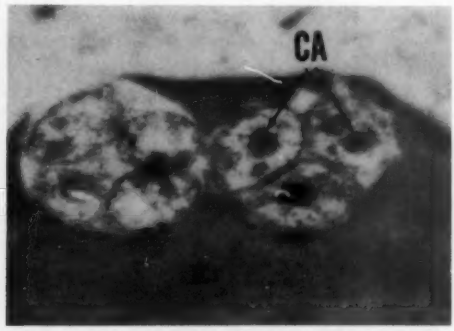

Fig. 3—Vestopal-W-embedded erythrocyte (Er) containing 2 Anaplasma bodies. The body at the right contains 3 small units, each with a dense central area (CA). The body to the left contains 4 small units. x 45,940

in diameter). Ristic [13] reported the initial bodies (elementary bodies) occupied approximately three fourths of the structure of the classical inclusion body (marginal *Anaplasma* body), and the initial bodies appeared laterally fused with a central undifferentiated mass. Our study, using sectioned erythrocytes, has shown as many as 7 small units (initial bodies of Ristic) in a marginal *Anaplasma* body with no central, undivided, or undifferentiated mass. We believe that this previously observed central material is produced in replicated or shadowed whole *Anaplasma* bodies by the collapse of the outer membrane about the inner small units. The situation is analogous to looking down on a flattened plastic ball containing smaller balls. It is thus not an intrinsic difference in marginal *Anaplasma* bodies we are dealing with, but the way one looks at them.

Projections or tails extending from the *Anaplasma* body and motility have been observed by Espana [8] in the phase microscope with hemolyzed infected erythrocytes. Ristic,[13] using infected erythrocytes, observed less than 3% of the marginal bodies to show passive vibratory movement during hemolysis. An oval projection was noticed with those bodies, showing vibratory movement that was interpreted to be matrix material of the *Anaplasma* body. Espana [8] also observed forms similar to those seen by phase microscopy with whole mounted erythrocyte stroma in the electron microscope. Such forms have not been reported previously by DeRobertis [7] or Ristic [13] in similar preparations. We have not observed evidence of projections or tails of marginal *Anaplasma* bodies in many sections of erythrocytes. It is difficult to resolve these conflicting reports, but an explanation may be found in the work of Brunner and Vallejo-Freire.[4] They found that guinea pig reticulocytes, hemolyzed by a variety of techniques, contained granules, circular forms, large filaments, and fine filaments, the varieties of which depended on the hemolytic technique employed. Filaments and circular forms in hemolyzed reticulocytes may explain the forms observed by Espana, and suggest that control observations be made on blood containing reticulocytes.

It is important to consider the embedding material when interpreting electron micrographs of sectioned cells. During methacrylate polymerization a reduction in volume occurs which can cause distortion.[1] The small dense particles in the 3rd form of the small unit of the *Anaplasma* body, previously interpreted as possible virus particles, are felt to be an artifact produced by rupture and coiling of filaments in the units. With vestopal-W, hardening begins slowly and terminates rapidly, lessening the chance of distortion.[11] *Anaplasma* bodies in vestopal-W generally showed better preservation than those in methacrylate, as evidenced by intact membranes and reduction in space between the units due to shrinkage. Fragility of the *Anaplasma* body may explain some of the variations in appearance in replicas and whole mounts in which the body has been subjected to drastic physical procedures.

The present study is an extension of work previously reported from this laboratory by Foote,[9] where small units were observed in the marginal *Anaplasma* body. More recent studies by Ristic [13] confirm the presence of small units (initial bodies) in the marginal inclusion (classical inclusion). The structure of the small units (initial bodies) described in this report vary somewhat from both the previous reports. Ristic [13] reported a limiting membrane with an internal undifferentiated mass containing numerous, dense, irregularly distributed, polymorphic particles approximately 10 to 20 mμ in diameter. Whether the polymorphic particles of Ristic are the same as the particulate material reported by Foote cannot be decided from published photographs. We have concluded that the particles in metha-

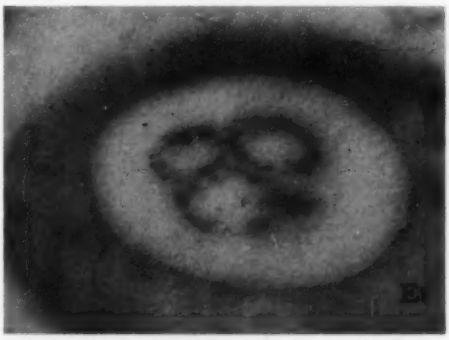

Fig. 4—Methacrylate-embedded, lead hydroxide-stained section of an Anaplasma body. The 3 small units of the Anaplasma body contain small dense particles. Compare the space about the small units with that in the other micrographs of cells embedded in vestopal-W. x 35,440

erylate-embedded infected erythrocytes are probably artifacts produced during polymerization of the plastic, as these are rarely found in vestopal-W embedded erythrocytes from the same animal. The present study with vestopal-W embedded erythrocytes clearly shows a limiting double membrane on the small units of the *Anaplasma* body enclosing a variable amount of amorphous granular or filamentous material. A published photograph of an *Anaplasma* body in a deer erythrocyte has shown an identical appearance.[2] There are no recognizable cell organelles in the *Anaplasma* body. The nature of the central dense mass in the 2nd form of the small unit is unknown. It is possible that the difference between the 1st and 2nd forms is due to the plane of section, but the similar size does not substantiate this. Histochemical techniques applied to sectioned cells in the electron microscope are needed to elucidate the nature of the contained material in the small unit of the *Anaplasma* body.

We have considered the *Anaplasma* body to consist of the small units contained within the space at the margin of the erythrocyte plus the membrane limiting the space. Whether the membrane is a part of the infective agent remains to be determined. This membrane is probably a part of the erythrocyte rather than the infective agent; the infective agent is probably the small unit within the larger *Anaplasma* body. Ristic [13] has concluded that the small unit (initial body) is probably the infective unit because it is present in erythrocytes of carrier animals.

An indisputable classification of *Anaplasma marginale* remains to be established, although possible categories can be narrowed. It is not a protozoan, since it contains none of the organelles of a cell. It is unlikely that it is a virus, as evidenced either by the size of the large body or of the small units. The most likely classification remaining is rickettsia. Few reports are available on the structure of rickettsia; Wissig [16] describes no rickettsial structure comparable to the structure of the *Anaplasma* body.

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SUMMARIO IN INTERLINGUA

Microscopia Electronic de *Anaplasma marginale* in le Erythrocyto Bovin

Studios del corpore de anaplasma per medio del microscopio electronic ha monstrate que ille corpore es componite de un membrana retenente que inveloppa plus micre unitates. Istos appareva in 3 formas morphologic. Le prime 2 formas differeva per le presentia o absentia

de un dense massa central. Le tertie forma, characterisate per numerose dense particulas in le micre unitates intra le corpore de anaplasma, esseva interpretate como un distortion artefactive de ille corpore.

Le resultatos del presente studio es discutite in le lumine de previe reportos con respecto al apparentia del corpore de anaplasma sub le microscopio electronic. Il pare que le corpore de anaplasma es ni protozoic ni virusal sed, probabilemente, rickettsial.

Correction—Spontaneous Abnormal Cardiac Arrhythmias in the Dog

The following information should have been contained in the footnote (p. 355) for the article "Spontaneous Abnormal Cardiac Arrhythmias and Conduction Disturbances in the Dog—A Clinical and Pathologic Study of 3,000 Dogs," by D. F. Patterson, D. K. Detweiler, K. Hubben, and R. P. Botts, which appeared on pages 355–369 in the May, 1961, issue (Vol. XXII, No. 88) of the *American Journal of Veterinary Research:* The work described in this report was supported by Research Grants H3541 and H4885 from the National Heart Institute of the National Institutes of Health.

Correction—Metabolic Aspects of Ruminant Ketosis

In the report "Metabolic Aspects of Ruminant Ketosis" by D. S. Kronfeld, which appeared in the May, 1961, *Am. J. Vet. Res.* (pp. 496–501), there are four errors.

On page 496, the sentence beginning in the tenth line of the first paragraph should read: Frank ketosis (characterized by readily detectable acetone and acetoacetate, as well as β-OH-butyrate)[37] does appear during bovine ketosis, ovine pregnancy toxemia, and in fasted pregnant ewes.

Also on page 496, in the ninth line of the second paragraph, the superscript (1) should be omitted.

On page 498, second column, sixth line from the bottom, the reference number should be 28; on page 500, first column, second line, the reference number should be 3.

Demonstration of *Anaplasma marginale* with the Fluorescent Dye, Acridine Orange; Comparisons with the Complement-Fixation Test and Wright's Stain

J. H. Gainer, D.V.M., M.S.

SUMMARY

The fluorescent dye, acridine orange, was successfully applied in a simple method for the demonstration of Anaplasma marginale in formalin-fixed erythrocytes. The best pH for staining was found to be 1, acidified with HCl. All particulate nucleic acid materials, including such cells as leukocytes and immature erythrocytes, took up the dye. The Howell-Jolly body stained as if it contained ribonucleic acid.

The fixation of blood cells by collection directly in 10% formalin-saline solution (a) hardened the erythrocytes so that they withstood the acid dye treatment, and (b) served as an excellent means of preserving the blood collected in the field for the acridine orange staining of A. marginale.

Three groups of blood samples representing 544 animals were compared by the acridine orange stain and the complement-fixation (CF) test. One of these groups contained a high percentage of animals under 1 year of age possessing infection as detected by the staining method without the presence of CF antibody; the correlation ranged from a low of 25.6% to a high of 61.6%.

INTRODUCTION

Various fluorescent dyes have been used to demonstrate many biological materials, including such things as plant cells, bacteria, vertebrate epithelium,[5] cancer cells,[3,4] malaria, trypanosomes,[5] and viruses.[1] Recently, much study has been devoted to acridine orange (AO)[1] which, at pH 1 to 3, differentiates deoxyribonucleic acid (DNA) from ribonucleic acid (RNA). The detection of *Anaplasma marginale* by the conventional Wright's or Giemsa's staining procedure presents doubts even for the experienced worker, especially where the percentage of infected cells is low and where dye deposits may be confused with the parasite. The current renewed interest in fluorescence microscopy and its advantage over conventional microscopy in the detection of cancer cells and in viral studies led to the consideration of the use of AO for the demonstration of *A. marginale*.

This report describes the method of demonstrating *A. marginale* with AO and gives preliminary data comparing the AO stain, Wright's stain, and the complement-fixation (CF) test.

Materials and Methods

Blood Samples.—Blood was obtained from cattle naturally infected with *A. marginale* in the acute stage, in the carrier stage as confirmed by the CF test, and as samples, essentially unknowns, submitted to the diagnostic laboratory for examination.

Fixation.—Method One.—Air-dried blood smears, thick or thin, were fixed at least 24 hours in 10% formalin-saline solution which consisted of 1 volume commercial formalin, 9 volumes distilled water, and 0.85% NaCl, by weight. The stock bottle of formalin-saline solution was neutralized by putting magnesium carbonate, suspended in the fixative, in a dialyzing bag inside the bottle. Slides were of good quality, clean, and free from scratches. Most darkfield condensers specify a certain slide thickness. Reichert* uv slides were of especially good quality and free from autofluorescence. Slides were rinsed in distilled water prior to staining.

Method Two.—Blood was collected directly from the animal in formalin-saline solution (*see* Method One), 1 volume of blood to 6 to 10 volumes of fixative. Ten-milliliter tubes were prefilled with 7 ml. of fixative; 1 ml. of blood was added. Contents

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From the Florida Department of Agriculture Animal Disease Diagnostic Laboratory, Kissimmee, Fla.

Mr. R. C. Andrews and Mr. G. W. Stroup were most helpful as technical assistants. Thanks are due all staff members of the Diagnostic Laboratory for their suggestions and encouragement; namely, Drs. W. L. Sippel, Chief, D. E. Cooperrider, E. M. Ellis, T. E. Murchison, J. W. Needham, and Mr. K. F. Schilling. The CF tests were kindly performed by Prof. Olin Kliewer, Oklahoma State University, Dr. M. Ristic, University of Illinois, and Dr. W. H. Martin, Beltsville, Md. Examinations of Wright's stains were performed by Dr. D. E. Cooperrider.

* Wm. Hacker & Co., West Caldwell, N.J.

were gently mixed. Fixation continued for at least 24 hours at room temperature. If the cells had not gravitated out, they were centrifuged at 1,000 r.p.m. for 5 minutes. The supernatant fluid was decanted; the chocolate-colored cells were resuspended in 2 to 3 volumes of physiologic saline solution or in the formalin-saline solution, by using an applicator stick, by vigorous shaking, or by placing the tightly stoppered tubes in a shaking machine. Ordinary thin blood smears, as with fresh blood, were made; or thick smears were made using an applicator stick to transfer a small amount of blood to a slide, making the smear about 0.5 cm. in diameter. Smears were air-dried. As many as 12–14 thick smears were included on one slide.

Staining.—Dye Preparation.—Stock acridine orange solution (2,8 *bis*-dimethylaminoacridine) was made by dissolving 1 Gm. of dye* in 100 ml. of 0.1N HCl. It was stored at 4 C. The working solution, 1:1,000, was 1 volume of stock and 9 volumes of 0.1N HCl solution, again stored at 4 C. (National Aniline Division lots varied somewhat in rapidity of solubility and color index but were satisfactory for conditions established at the outset; at least one other manufacturer's lot was not satisfactory for conditions developed for the National Aniline product.)

Staining Procedure.—Smears were placed in the working solution for 10 seconds and were rinsed in 3 changes of distilled water or until no more dye washed from the slide. The stained smears were air-dried or blotted dry with new bibulous paper or a new paper towel. No cover slips were used.

Equipment for Examination.—A bright ultraviolet source or near ultraviolet source is essential. The Reichert 200-watt Osram high-pressure mercury-vapor arc lamp and filter system was used in this study. The exciter filter was the BG 12 Light pass filter, transmittance wave length of 312 to 509 mμ, peak at 405 mμ. A cardioid darkfield condenser was used in a monocular microscope, a 97x oil-immersion objective with funnel stop or a 40x oil objective, and a 5x to 25x ocular. Barrier filters were the Wratten G gelatin filter in the tube of the microscope and the Reichert OG 1 over the eyepiece; these filters were used separately or together. Fluorescent-free oil was used between condenser and slide and between slide and objective; glycerin may be used as the immersion medium.

Duplicate smears were made of some samples, stained with Wright's stain, and examined by ordinary light microscopy. Serums were preserved with phenol and mailed to Oklahoma State University or the University of Illinois for CF testing.

Results

The accompanying figures (Fig. 1–5) illustrate the fluorochroming of the formalin-fixed blood with AO. (All smears illustrated were stained 10 sec. in 1:1,000 AO, pH 1.0; magnification is x1,000.) The typical marginal bodies appeared as brilliant orange spots at the periphery of the faint green erythrocytes. In addition to the typical marginal bodies or in the absence of the typical marginal bodies, small green struc-

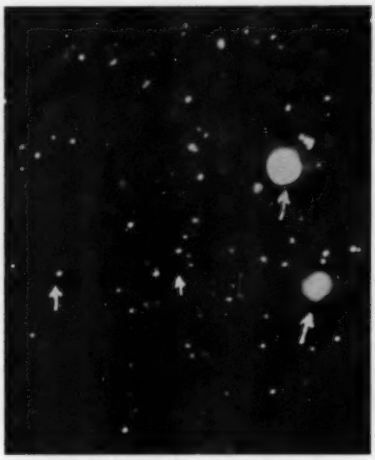

Fig. 1—Thin smear, high percentage of erythrocytes infected; whole blood fixed in formalin-saline solution. Left arrow, 1 parasite per cell; middle arrow, 4 parasites per cell; right arrows, lymphocytes. Some cells contain only small forms.

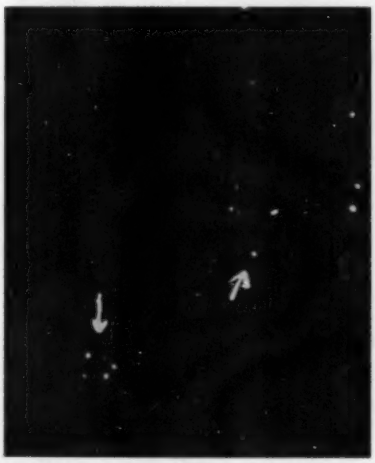

Fig. 2—Thin smear, moderate percentage of infected cells; smear was air-dried one year, then stained. Left arrow indicates several parasites per cell; right arrow indicates 1 typical marginal body, 2 small bodies.

* National Aniline Division, Allied Chemical, New York, N.Y.

TABLE 1—Comparison of Acridine Orange Stain with the Complement-Fixation Test

Group	No. animals	AO+CF+	AO+CFs	AO+CF−	AOsCF+	AOsCF−	AO−CF+	AO−CFs	AO−CF−	% correlation
1	33	7	1	4	0	0	12	0	9	48.5
2	39	23	3	3	1	1	6	1	1	61.6
3	472 (Total)	136	22	201	2	7	38	5	59	41.8
	390 (Adult)	131	21	147	2	3	36	5	45	45.1
	82 (1 yr. & less)	7	1	54	0	4	2	0	14	25.6

Groups 1 and 3—CF by Prof. Kliewer; group 2—CF by Dr. Martin.

tures were seen occasionally. Lymphocytes and other monocytic leukocytes fluoresced a brilliant orange throughout the cell. Neutrophilic cytoplasm was green, and its granules were a brilliant orange. Thrombocytes stained a light yellowish-green to a faint orange-red. Bacteria stained either red to orange or green. Howell-Jolly bodies appeared brilliant orange. Immature erythrocytes fluoresced a dull, diffuse orange. Plasma proteins appeared as light green homogeneous material between the cells.

Table 1 summarizes some preliminary information comparing the AO stain and CF test. Group 1 consisted of 12 accessions as they came through the diagnostic laboratory. One infected cell, observed in a 0.5-cm. smear consisting of 20 to 25 fields that comprised 10,000 to 20,000 cells, was recorded as AO positive. Smears in which structures suggestive of marginal bodies, but in which absolute certainty as to their being marginal bodies was questioned, were considered AO suspect. If no infected cells were seen in the whole smear, the sample was AO negative. Group 2 consisted of 2 accessions from experimental herds in Mississippi. Fourteen animals had been on antibiotic therapy. Positive CF titers ranged from 1:5 to 1:80 in 24 animals; in 13 animals, 1:100 was the lowest dilution conducted.

Fig. 3—Whole blood fixed in formalin-saline solution for the detection of the carrier state of anaplasmosis; no marginal bodies present. Top arrow illustrates a Howell-Jolly body; bottom arrow illustrates fluorescence of soluble plasma constituents absent in thoroughly washed smears. Erythrocytes are somewhat irregular due to fixation. Irregular white objects throughout are platelets; the large white cell is a leukocyte. Notice that the diameter of the Howell-Jolly body is nearly twice the diameter of Anaplasma bodies in other figures.

Group 3 (Table 1) represents a herd of dairy cattle of all ages, in central Florida; among these animals there was a high percentage of clinical disease during the fall of 1959. The blood samples were taken in late April and early May, 1960. The CF tests, conducted by Prof. Kliewer, left much to be desired in interpretation in view of anticomplementary effects caused by phenol preservation. However, some information may be gained by including the results. The 7 anticomplementary samples were not included in the table. When the herd was allotted to two groups, one of animals 1 year and under, and one of adults, the percentages in the younger group varied considerably more than in the adults. The greatest variation occurred in the AO+, CF−, where the proportion was 54/82 (65.8%). Two calves, 1 month old, possessed CF titers, probably maternal antibody, but at the same time they were positive by AO staining.

Wright's stain, used on smears from 23 of 33 animals in group 1, showed 6 positive and 17 negative; in group 2, 7 of 39 were positive.

Discussion

It has been shown by several workers[1,2,7] that AO combines specifically with the nucleic acids (NA). The dye has been called polychromatic in that when present in high concentration, it fluoresces orange, and in

low concentration, green. The exact nature of the differential fluorescence of AO–NA may not yet be satisfactorily explained, but to date the evidence appears as follows: (1) In the orange fluorescence observed with staining of ribonucleic acid (RNA), Beers[2] states that linkages (complex I) involve the bases and internucleotide phosphates of polyribonucleotides; whereas (2) with desoxyribose nucleic acid (DNA) which stains green, the linkages (complex II) involve only the terminal phosphates of both polyribonucleotides and DNA. The DNA then forms only complex II, and therefore always stains green.

In theory at least, AO should be a good stain for *A. marginale* for several reasons: (1) The mature mammalian erythrocyte contains no nucleic acid.[10] (2) The immature erythrocyte containing NA holds it as RNA only in a diffuse state.[10] (3) *A. marginale* contains both nucleic acids.[6] (4) Leukocytes remaining in a smear with erythrocytes might act as a constant check for proper staining results since they have both nucleic acids. The photographs tend to bear out these assumptions. The typical marginal body contains both nucleic acids; it stains orange with AO because it RNA overshadows the DNA. The small, green forms may be early developmental ones containing only DNA. Thrombocytes, although reportedly void of RNA,[10] stain with AO as if they do contain some NA, or else they are highly buffered and their protein carboxyl groups are combining in high ratio with the AO. The only possible confusion which might exist is in differentiating *Anaplasma* bodies from Howell-Jolly bodies. They have been reported void of DNA,[8] but whether they contain RNA seemingly has not been reported. Findings here suggest that they do contain RNA. Their size being 2 or more times larger than the *A. marginale*, and their central or eccentric position in the erythrocyte rather than the peripheral position of the *Anaplasma* body, should differentiate the 2 structures.

Although the amount of data is small and subject to some doubt as to its reliability, comparison of the AO stain with the CF test offers encouragement for use of the stain in demonstrating carriers that were formerly recognized only by means of the CF test, and rarely, if ever, by the conventional Wright's and Giemsa's stains. The AO stain seems particularly useful in demonstrating carriers that possess high CF titers; whether

Fig. 4—Whole blood fixed in formalin-saline solution, thin smear. Carrier state by CF test. This typical marginal body was found only after examining many fields, from 10,000 to 20,000 cells. Marginal body is at the point of the arrow.

it is sufficiently good to detect carriers with low CF titers remains to be determined. The AO stain theoretically should be better in detecting the infected animal than is the CF test because with the AO procedure one examines for the presence of the organism. The CF test depends upon the presence or absence of antibody, and such antibody may be absent or of such low level as to be indetectable. The reliability of AO is predicated on the assumption that the carrier animal possesses organisms in its circulating peripheral blood—a condition which exists naturally, otherwise dissemination of the disease would not occur as it does. Final judgment on the efficacy of AO will

Fig. 5—Whole blood fixed in formalin-saline solution, thick smear. CF results are positive. White objects of irregular shape and size are platelets. Erythrocytes are somewhat irregular in shape due to fixation. Marginal body at point of arrow.

rest upon challenge of suspect blood in susceptible animals. Such challenges are yet to be performed.

The presence of a high percentage of infected young animals in a naturally infected herd in the absence of CF antibody is not readily explainable. The condition known as immunologic unresponsiveness or acquired immunologic tolerance may be operating.

The demonstration of small green particles, possibly DNA-containing forms, barely resolvable with the fluorescence microscope, in some samples in rather high percentages, suggests similarities to Dr. Ristic's[9] findings with electron microscopy in that a high percentage of small forms are seen in carrier animals. The AO stain certainly appears to be much more reliable in detecting carriers than does the Wright's stain.

The nature of fluorescence microscopy, that is, that a bright object stands out significantly better from a dark background than does just a different colored object from its background in visible light microscopy, readily yields results where conventional microscopy fails. The specific nature of AO itself for nuclear material and its lack of confusing dye precipitates is helpful. One cell containing an *Anaplasma* body may be recognized out of 10,000 or more normal cells. Although the stain admittedly is not specific as is the fluorescent antibody method, the size and position of the parasite within the red blood cell makes it specific.

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SUMMARIO IN INTERLINGUA

Demonstration de *Anaplasma marginale* per Medio del Colorante a Fluorescentia, Orange Acridina; Comparation con le Test a Fixation de Complemento e le Tincturation de Wright

Le fluorescente colorante orange acridina esseva applicate a bon successo in un simple methodo pro le demonstration de *Anaplasma marginale* in erythrocytos fixate per formalina. Esseva trovate que le optime pH pro le tincturation es 1, acidificate con HCl. Omne particulate materiales a acido nucleic, incluse cellulas como leucocytos e erythrocytos immatur, acceptava le colorante. Le corpore de Howell-Jolly se tincturava como si illo contineva acido ribonucleic.

Le fixation del cellulas de sanguine per colliger los directemente in un solution salin de formalina de 10% (a) indurava le erythrocytos de maniera que illos supportava ben le tractamento con le colorante acide e (b) serviva como un excellente methodo pro preservar le sanguine colligite in le campo pro le tincturation a orange acridina de *A. marginale*.

Tres gruppos de specimens de sanguine, representante un total de 544 animales, esseva usate in un comparation del tincturation a orange acridina con le test a fixation de complemento. Un de iste gruppos representante un grege lactifere sub studio habeva revelate un alte procentage de animales de etates de minus que un anno con absentia de anticorpore a fixation de complemento. Le correlation in iste gruppo particular esseva 25,6% e attingeva 61,6% in un del alteres.

The Measurement of Bovine Thyroid Activity, Using Low Dosages of Radioactive Iodine I^{131}

J. R. Howes, Ph.D.; W. A. Higinbotham, Ph.D.; J. F. Gennaro, Ph.D.; J. P. Feaster, Ph.D.

SUMMARY

An instrument making it possible to measure effectively the bovine thyroid uptake and release of I^{131}, using relatively low doses, was so designed that the signals from 2 scintillation probes could be read on one ratemeter. Such a device provides greater efficiency than the conventional 1-probe apparatus in the detection of emission from radioactive isotopes in the animal body, not only because of the increased surface area afforded by the 2 probes, but also because of the possibility of positioning for greatest geometrical advantage.

Small-dose uptake in the thyroid glands of cattle was used to demonstrate the usefulness of the apparatus, since hitherto relatively large doses of I^{131} have been employed for this purpose. Doses of from 1 to 10 μc. were successfully used, whereas 100 and 300 μc. are usually required. The problem of confusing thyroid function data with radiobiologic effect was considerably reduced, because the radiation dose to critical tissues was less.

INTRODUCTION

In a relatively long-term comparative physiologic study with cattle, a concern was the possibility of thyroid damage of both an immediate and permanent nature to experimental animals. Although the radiobiologic effects encountered by Bustad *et al.*,[5,6] who continually administered I^{131} to sheep for 4 years, are not envisaged, other less apparent biologic abnormalities could occur.

A survey of the literature concerning the use of I^{131} in the study of thyroid metabolism of domestic animals reveals, in general, the use of relatively high doses of I^{131}. Especially is this so in work carried out with cattle given doses of 0.1 to 20.0 mc.[2-4, 14, 15, 19-22, 27, 28, 32, 33]. Recently, there has been a report describing the use of 50 μc. as a lower limit of dosage.[23]

In most higher mammals, the thyroid gland consists of 2 lobes connected by a narrow isthmus. It has become a common practice to use a single scintillation probe to determine the I^{131} in this bilobed gland. The coupling of 2 Geiger-Mueller or 2 scintillation probes in parallel to overcome geometrical problems in the measurement of I^{131} uptake by the human thyroid gland has been recently described, but without any details of the circuits used.[10,12] It has also been demonstrated that I^{131} in the thyroid gland is not homogeneously distributed, as has been assumed.[1,16] For these reasons, the authors believe that a single scintillation probe is not the best instrument to measure accurately the I^{131} retained in the thyroid gland.

Test *et al.*[34] devised for pigs an array of 3 probes located radially 120 degrees apart about the longitudinal axis of the animal. This design proved awkward and difficult to reposition, so a 2-probe unit with probes diametrically opposite was finally used.[29] The instrument used in the present study consisted of 2 scintillation probes which simultaneously fed signals into 1 ratemeter. The possible applications of such an instrument to the measurement of radioactivity are many; one study with laboratory animals has already been reported.[13]

Materials and Methods

Apparatus.—The instrument consisted of 2 cylindrical, shielded scintillation probes, a preamplifier, a ratemeter, and a recording milliammeter. For work with cattle, a restraining chute with a

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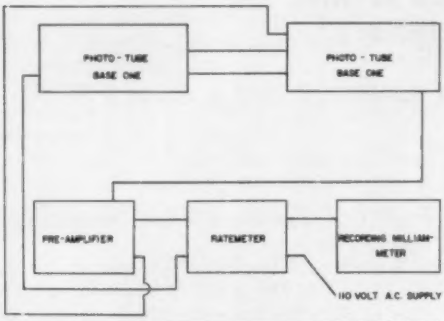

Fig. 1—Schematic diagram of the apparatus, showing connections between the 2 photomultiplier tube bases, preamplifier, ratemeter, recording milliammeter, and power supply.

metal frame attached for securing the animal's halter was necessary. The 2 scintillation probes (1½ inch diameter x 1 inch NaI crystals) were fitted to a wooden frame so that they inclined toward each other at an angle of about 45 degrees. The open unshielded ends of the cylindrical probes fit close against the sides of the neck of the restrained animal, thereby covering both lobes of the thyroid gland and minimizing geometrical and collimation errors. The distance between the ends of the probes and the thyroid gland is small and fairly constant. The height of the scintillation apparatus and the restraining frame was adjustable.

Figure 1 shows the method by which the 2 photomultiplier tubes were connected with the preamplifier, ratemeter, recording milliammeter, and 110 volt A.C. power supply. The circuit for the photomultiplier tube base (Fig. 2) and the preamplifier circuit (Fig. 3) are shown. Most available scalers and ratemeters have a 0.25-volt input sensitivity, but this preamplifier has a gain of 30, giving an input sensitivity of about 0.01 volts, which is optimum for use with a scintillation counter. The preamplifier may be located close to the detectors so that the signal at the photomultipliers is not appreciably attenuated by cable capacitances. The advantages of this circuit have been described.[7] The "fine gain controls" (Fig. 2) were used to match the gains of the 2 photomultipliers. Figure 4 illustrates the equipment.

Biologic Technique.—To evaluate the described apparatus, 6 adult, nonpregnant Hereford cows of approximately the same age and weight were selected for treatment with I^{131}. They had access to pasture and water only. For dosage, the 6 cows were allocated at random to 3 pairs, and the cows in each pair were given 1 of 3 experimental dose levels of I^{131}; *i.e.*, 1, 5, and 10 μc. Injection was by way of the left jugular vein. At various intervals of time thereafter, the cows were placed in the restraining chute for measurement of the thyroidal I^{131} activity, care being taken to place the probes immediately over the thyroid gland, as indicated by a maximum reading on the count-rate meter. Three 1-minute tracings were made on the recording milliammeter on each occasion for each cow, and the 3 maximal values or peaks from these tracings were averaged to provide the mean count.

Background counts were made before and after each series of readings, and the mean background was subtracted from the mean count made on the thyroid gland to give the corrected count. On the 1st day, just prior to injection of the isotope,

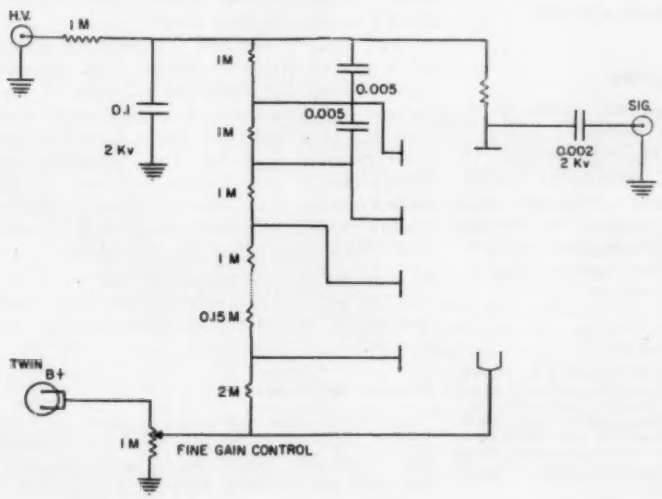

Fig. 2—The circuit diagram of a photomultiplier tube base.

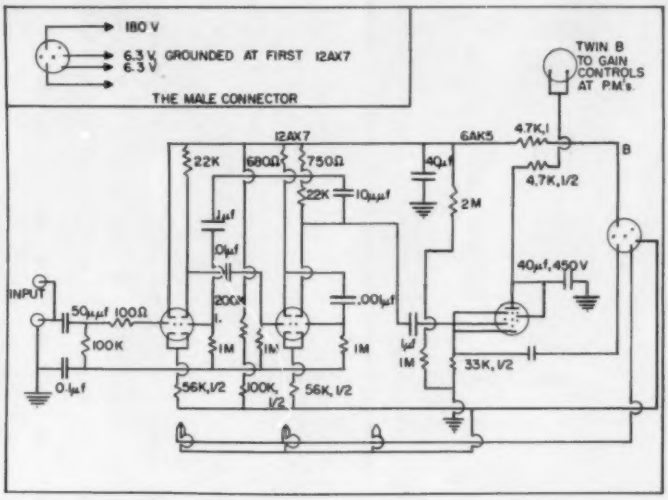

Fig. 3—Preamplifier circuit diagram (P.M.= photomultiplier tubes).

a volumetric flask about the size of the bovine larynx and containing a dose identical to that given each cow was monitored with the same equipment. The readings obtained were averaged and the mean value, after correcting for background, provided a standard estimate of each dose in

Fig. 4—Front view of the equipment used to measure I^{131} uptake by the thyroid gland of a restrained Brahman heifer. The 2 probes inclined toward each other on the wooden support have been drawn to one side for a better view.

TABLE 1—The Mean Counts per Minute, in Thousands, Corrected for Background and Radioactive Decay, and the Percentage of the Dose of I^{131} Taken Up by the Thyroid Glands of Each of Three Pairs of Heifers

I^{131} (μc.)	Hours after injection															
	6		24		48		72		96		168		264		408	
	C/M	%	C/M	%	C/M	%	C/M	%	C/M	%	C/M	%	C/M	%	C/M	%
1	2.9	12	5.1	21	7.3	30	15.1	63	12.3	51	11.1	46	1.3	5	0.5	2
5	1.6	3	9.3	15	12.0	20	17.6	29	14.5	24	11.5	19	12.9	21	11.7	19
10	10.8	10	20.6	20	33.2	32	50.1	48	36.7	35	30.0	29	34.1	33	26.0	25

counts per minute. No phantom was used with the described equipment, since the error at such a close counting distance was small.[17]

Subsequent *in vivo* thyroid counts were corrected for background and radioactive decay. In order to obtain the percentage of the injected isotope remaining in the thyroid gland after various intervals of time, the corrected *in vivo* counts were expressed as percentages of the injected isotope in counts per minute.

Results and Discussion

The results obtained from the 6 animals given doses of I^{131} from 1 to 10 μc. are presented (Table 1, Fig. 5). It can be readily seen that the time of maximum uptake of I^{131} was 72 hours after injection, about midway in the range of values for the maximum uptake time in European cattle reported by Blincoe and Brody.[4] The thyroid uptake and release curve (Fig. 5) was much the same at all dosage levels, the variation between the 3 curves possibly being due to the small number of animals used. However, it has been demonstrated that the trapping of I^{131} by the thyroid gland appears not to be greatly affected by dosage level; rather, there is interference in the formation and the release of thyroxine by the gland.[11]

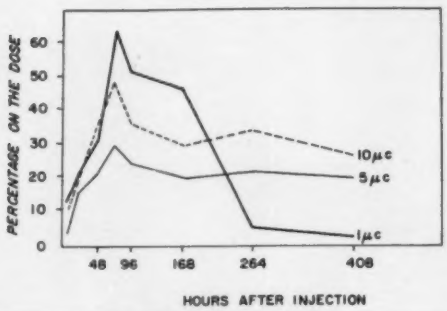

Fig. 5—The mean percentage of the dose of I^{131} in the thyroid gland at various intervals after injection.

In another study, 24 adult cattle each were given 5 μc. of I^{131} intravenously. The results for 1 of the 4 treatment groups of 6 heifers are presented (Fig. 6). The graph shows the percentage of the I^{131} dose remaining in the thyroid gland at various intervals after injection for 6 Hereford heifers similar in age and weight to those used in the trial reported here. The slope of the curve is less erratic since there are more animals involved.

The advantages of small doses of I^{131} are obvious if it is remembered that even relatively low doses can be hazardous because of the thyroid concentration of I^{131}. According to standards for man agreed upon internationally, the basic maximum permissible dose to a critical tissue from *beta* and *gamma* rays of internally distributed radioisotopes is 0.3 rad per week,[16,18,25] though this value has been questioned by Morgan.[24] The concentration which delivers 0.3 rad per week can

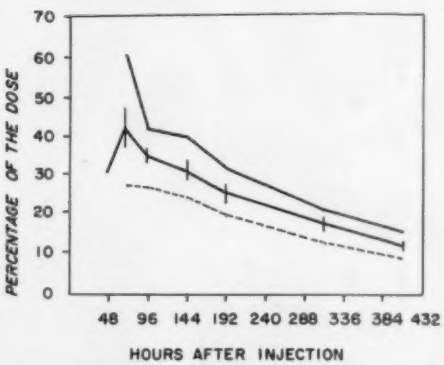

Fig. 6—The mean percentage of the dose of I^{131} in the thyroid gland at various intervals after injection for 6 heifers, each given 5 μc. The upper and lower lines represent the maximum and minimum values. The center line represents the mean, and the vertical lines indicate the standard error of the mean.

be calculated [16*] as being 0.0061 μc. per gram of tissue if it is assumed that the combined lobes of a bovine thyroid gland may be ideally represented by a sphere of radius 2 cm.[31]

If the weight of a typical adult bovine thyroid gland is assumed to be 20 Gm.,[31] and 50% of the injected iodine is taken up by the gland, then it is possible to calculate the concentrations of isotope in μc./Gm. of thyroid tissue for any given level of injected I^{131}. For the 100 and 50 μc. levels frequently used in bovine studies, the concentrations of I^{131} in the thyroid gland are 2.50 and 1.25 μc./Gm., respectively. The need for lower doses than those used at present with cattle seems obvious. The possible use of I^{132} with a half-life of 2.26 hours renders this isotope valuable for clinical studies as has been indicated by Cook *et al.*[8] However, it is doubtful whether this potential method of reducing radiation hazard is applicable for work with large animals, because it requires 2 to 4 days to achieve the maximum uptake of injected iodine.

The appearance of some thyroid adenomas have been reported recently in children and teenagers 5 to 10 years after radioiodine therapy, but no instances have as yet been found in patients who were adult at the time of treatment.[30] This suggests that the thyroid tissue of growing animals might be more radiosensitive than that of adults. Some workers [9] indicate that I^{131} irradiation of the thyroid gland causes a prolonged rise in secretion of thyroid-stimulating hormone (TSH). This effect has been produced with doses of as low as 30 μc. of I^{131}. Thus, I^{131} not only appears to initiate neoplastic thyroid cells, but also promotes tumor formation by its effect on TSH secretion.

In view of these considerations, it is imperative that much lower doses of I^{131} be used for work with large animals. This study has indicated that a dose of as low as 1 μc. of I^{131} can be satisfactorily employed in thyroid studies with adult cattle weighing 1,000 lb. These lower doses greatly reduce the possibility of confounding radiobiologic effect with thyroid function. In order to employ such small doses with such large animals, a sensitive monitoring instrument is essential. One such device has been described.

Research workers considering studies with livestock in which radioisotopes are to be used are reminded of the ruling of the Food and Drug Administration barring the use of food derived from animals that have been subjected to treatment or diagnosis with radioactive isotopes.

* Equation 95 from page 863 [16] is:

$$S = \frac{4.1 \times 10^{-8}}{(E_B + 4.7 \times 10^{-4} \Gamma \bar{g})\ T_{eff}\ (1 - e^{-4.85/T_{eff}})}$$

$$\frac{\text{microcuries}}{\text{gram}}$$

Where E_B is the average *beta* ray energy (for $I^{131} = 0.187$ Mev.), Γ is the point source *gamma* ray dose rate (for $I^{131} = 2.18$ CM^2-r/mc-hr) $\bar{g}$ is the geometrical factor for the average *gamma* ray dose rate and Teff is the effective half life of the radioisotope (7.6 days for I^{131} in this study).

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SUMMARIO IN INTERLINGUA

Le Mesuration del Activitate del Thyroide Bovin, con le Uso de Basse Doses de Radioactive Iodo^{131}

Un instrumento permittente mesurar efficacemente le acceptation e liberation de I^{131} in le thyroide bovin con le uso de relativemente basse doses del elemento esseva construite de maniera que le signales de 2 sondas de scintillation poteva esser legite in un sol mensor. Un tal instrumento provide plus alte grados de efficacia que le apparato conventional a sonda unic in le detection de emissiones ab isotopos radioactive in le corpore animal, non solmente a causa del plus grande superficie controlate per le 2 sondas sed etiam a causa del possibilitate de positionar le sondas con le objectivo del plus grande avantage geometric.

Le acceptation de miere doses per le glandulas thyroide de bestial esseva usate pro demonstrar le utilitate del apparato, in vista del facto que usque nunc relativemente grande doses de I^{131} ha essite empleate pro iste objectivo. Doses de inter 1 e 10 μc esseva usate a bon successo, durante que inter 100 e 300 μc es usualmente requirite. Le problema del confusion de datos de function thyroidic con effectos radiobiologic esseva reducite considerabilemente in consequentia del facto que le dose de radiation recipite per tissus critic esseva plus basse.

Anthelmintic Activity of an Organic Phosphate in Cattle and Sheep

James F. Landram, M.S., and Robert J. Shaver, Ph.D.

SUMMARY

The reduction in helminth egg counts, the recovery of worms passed in the feces after treatment, and the efficacy obtained in critical studies illustrated that 4-tert-butyl-2-chlorophenyl methyl methyl-phosphoramidate has good anthelmintic properties for gastrointestinal parasites. Dosages of 40 mg./kg. or above in cattle and 75 mg./kg. or above in sheep were shown to provide good to excellent parasite control. The results of limited trials wherein the compound was mixed in the feed indicated that the material is palatable and effective by this method of application.

INTRODUCTION

There have been several reports of the anthelmintic activity of various organic phosphorus compounds. Crenshaw [2] found that ronnel * was effective against *Ostertagia, Haemonchus,* and *Cooperia* in cattle at a dosage of 125 mg./kg. Herlich and Johnson [6] reported the efficacy of ronnel as determined by critical studies.

The anthelmintic activity of Bayer L 13/59 was reported by Riek and Keith,[9] Levine *et al.*,[8] Gordon,[5] Galvin *et al.*,[4] and Stampa.[10] The latter 3 authors also reported that anthelmintic activity was obtained with Bayer 21/199 in cattle and sheep. Herlich and Porter [7] found Bayer 21/199 effective against several genera of parasites infecting the gastrointestinal tracts of cattle and sheep.

Baker *et al.*[1] and Douglas *et al.*[3] reported DOWCO 105 to be an effective anthelmintic in cattle and sheep and discussed indirect evidence of the possible activity of this material against the immature stages of some of the parasites.

A new organic phosphate, 4-tert-butyl-2-chlorophenyl methyl methyl-phosphoramidate,** has been shown to have anthelmintic activity against gastrointestinal nematodes of cattle and sheep. The following data were collected during the investigations of the activity of this product.

Materials and Methods

The chemical was formulated either as a 25% wettable powder and given orally in water or as the unformulated material given in gelatin capsules. When given in the daily feed ration, it was formulated as a 10% premix in soybean meal. All dosages reported in the following trials are expressed in milligrams of intake of the active ingredient per kilogram of body weight.

Nematode eggs per gram of feces (e.p.g) were determined by a zinc sulfate centrifugal flotation technique.

Methods pertaining to particular trials will be given under the appropriate section.

Results and Discussion

Trial 1.—Twenty-six commercial-grade crossbred cattle were purchased in August, 1958, at a local auction in Texas and were individually treated with various dosages of the organic phosphate to determine anthelmintic activity as evidenced by e.p.g. reductions (Table 1).

The e.p.g. counts were made on the 3 consecutive days prior to treatment and on the 6th, 7th, and 8th days after treatment. In some cases, samples of feces collected 48 hours after treatment were found to contain large numbers of nematodes of the genera *Haemonchus, Trichostrongylus, Ostertagia,* and *Cooperia.* Some of the animals were maintained on concrete floors, and e.p.g. counts for these were made 14 and 21 days after treatment. There was little or no change from the counts taken 1 week after treatment. Al-

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From the Dow Chemical Company, Lake Jackson, Texas (Landram) and Midland, Mich. (Shaver).

* Trolene (DOW-ET-57), Dow Chemical Co., Midland, Mich.

** Ruelene, Dow Chemical Co., Midland, Mich.

TABLE 1—Per Cent Reductions in Eggs per Gram of Feces, Resulting from a Single Oral Treatment of Cattle

Calf No.	Weight (lb.)	Active material given (Gm.)	Dosage (mg./kg.)	Av. % reduction in e.p.g. 6, 7, and 8 days post-treatment
650	350	3.2	20	78
654	350	3.2	20	54
656	262	2.4	20	70
657	304	2.8	20	43
658	298	2.7	20	27
662	370	5.0	30	99
663	427	5.8	30	95
666	371	5.0	30	88
669	410	5.6	30	96
670	300	4.1	30	99
524	346	7.8	50	44
525	356	8.1	50	96
577	325	7.4	50	92
578	350	7.9	50	99
580	314	7.4	50	99
587	417	9.6	50	96
563	324	7.4	50	88
565	384	8.7	50	95
573	342	7.8	50	99
589	382	8.6	50	99
569	362	8.1	50	96
519	294	10.0	75	63
520	232	8.0	75	89
480	156	5.0	75	95
490	290	13.2	100	96
325	252	11.0	100	100

though some activity was noticed at all dosage levels, it appeared that a dosage of 30 mg./kg. or higher would be necessary to attain acceptable anthelmintic control.

Trial II.—Seven locally purchased crossbred cattle were given the organic phosphate, formulated as a 25% wettable powder, to determine the minimum toxic dosage.

Two of 5 cattle given 150 mg./kg. developed severe signs of toxicosis within 3 hours after treatment. Both animals were recumbent, bloated, and dyspneic. Therefore, atropine sulfate therapy was begun. Both animals were able to rise at 13 hours after treatment, although ataxia, salivation, and general weakness were evident for 48 hours, after which time there was a gradual improvement until both animals appeared normal by the 10th day after treatment. The other 3 calves given the 150 mg./kg. dosage salivated and were slightly ataxic for 24 hours after treatment, after which time toxicosis was not evident.

Of 2 calves given 200 mg./kg., 1 salivated moderately and was slightly ataxic 24 hours after treatment. In the other animal there were no signs of toxicosis.

Trial III.—Four calves, weighing 200 to 350 lb. and naturally infected with nematode parasites, were maintained in the large-animal building at Lake Jackson, Texas, for one week. Then they were treated orally with the compound at 30, 40, 50, and 75 mg./kg., respectively. Critical evaluation of the efficacy of the drug was made by a modification of the method described by Swanson *et al.*[11] The calves were slaughtered 5 days after treatment, and the results are reported (Table 2).

Trial IV.—The results of a comparative anthelmintic field trial in cattle conducted at Brazoria, Texas, during July, 1958, are presented (Table 3). Fecal samples were

TABLE 2—Results of Critical Studies on 4 Calves Treated Orally

	30 mg./kg.		40 mg./kg.		50 mg./kg.		75 mg./kg.	
Worm species	% Efficacy	Total No. worms represented	% Efficacy	Total No. worms represented	% Efficacy	Total No. worms represented	% Efficacy	Total No. worms represented
Haemonchus	100	25	100	1	100	299	100	121
Ostertagia	100	50	96	1,050	100	600	90	2,660
Trichostrongylus	100	50	.....	.....	60	2,320	39	8,160
Cooperia	96	9,410	100	7,700	89	4,020	100	7,220
Oesophagostomum	0	52	47	15	50	184	100	17
Bunostomum	18	38	4	174	31	74	100	1

TABLE 3—Comparative Anthelmintic Field Trial with Cattle

Group	No. cattle	Treatment	Dosage of active ingredient in mg./kg. (range / average)	Dosage form	% reduction of e.p.g. 14 days post-treatment (range / average)
I	30	Organic phosphate	36–57 / 43	Capsule	88–100 / 98
II	30	Organic phosphate	48–67 / 57	Drench	0–100* / 98
III	28	Organic phosphate	33–52 / 43	Drench	83–100 / 98
IV	11	Phenothiazine	194–320 / 235	Drench	37–99 / 88

* The feces of 1 animal contained no parasite eggs prior to treatment and 4 e.p.g. following treatment.

taken at the time of treatment and 14 days after treatment. All calves were marked for individual identification and weighed at the time of treatment. The weights ranged from 315 to 460 lb., with an average of 385 lb., and the average e.p.g. count at the time of treatment was 240.

The calves were separated into 4 groups, and all those in a group were given the same dose. Those in group I (30 calves) were given 7.5 Gm. of the unformulated anthelmintic in gelatin capsules, those in group II (30 calves) were given 10 Gm. formulated as a 25% wettable powder, and those in group III (28 calves) were given 7.5 Gm. formulated as a 25% wettable powder. Calves in group IV (11 calves) were given 40 Gm. of N.F. green phenothiazine (*see* Table 3 for dosages given and results obtained).

The feces of a treated calf in group II contained no eggs before treatment and 10 eggs after treatment. Had this animal not been included, the range of e.p.g. reductions in group II would have been 83 to 100%.

Trial V.—In July, 1958, a field trial with sheep was conducted on a ranch near Boerne, Texas. The trial included 70 purebred Columbia lambs which were being maintained in 4 feeding pens. Each lamb was identified by a number tattooed in one ear. Individual fecal samples were obtained at the time of treatment and on the 12th day after treatment. Weights were not measured during these trials, and the average dosages reported were based on the estimated weights. The treatments and results are given (Table 4, groups I–IV).

Of the 20 lambs in group III, given 2 Gm. of the compound mixed in the feed, 6 had no reduction in e.p.g., 4 had reductions of 38 to 70%, and 10 had reductions of 83 to 100%. The 20 lambs in group IV were given 4 Gm. of the compound mixed in the feed. One lamb had no reduction in e.p.g.; one had a reduction of 36%; and the remaining 18 lambs had reductions

TABLE 4—Anthelmintic Efficiency in a Field Trial in Sheep

Group No.	No. sheep	Dosage per animal	Form	Av. estimated weight (lb.)	Av. dosage (mg./kg.)	Av. % reduction in e.p.g. 14 days post-treatment
I	15	3 Gm.	Drench	80	83	94
II	15	8 Gm.	Drench	100	178	98
III	20	1 Gm. in feed given twice, a.m. and p.m.	10% premix	65	34 x 2	40
IV	20	2 Gm. in feed given twice, a.m. and p.m.	10% premix	75	59 x 2	83
V	25	4 Gm.	Drench	60	148	95
VI	23	6 Gm.	Drench	100	133	97
VII	30	6 Gm.	Drench	165	80	90
VIII	30	8 Gm.	Drench	165	106	100

TABLE 5—Anthelmintic Efficiency in Lambs Treated with 75 mg./kg.

Group No.	No. lambs	Av. post-treatment % e.p.g. reduction		
		6 days	15 days	35 days
1	5	92.2	97.2	98.6
2	5	92.0	88.2	77.0
3	5	97.6	93.6	95.2
4	5	97.0	95.8	93.8
5	4	97.5	97.3	98.5
6	5	96.0	95.8	96.4
Average		95.3	94.7	93.3

greater than 82%. The organic phosphate given in this trial was formulated as a 10% premix in soybean meal. There was no indication that the addition of the compound affected the palatability of the grain ration.

Trial VI.—Another trial was undertaken in September, 1958, at the same ranch near Boerne, Texas. Sixty Columbia ewes on pasture and 48 lambs in a feedlot were allotted to 4 groups (V–VII) and given the compound as a drench. Those in group V were each given 4 Gm. of the compound; the weight range in this group was estimated as 40 to 80 lb. Those in group VI were each given 6 Gm. of the anthelmintic, and the estimated weight range was 80 to 110 lb. Both of these groups of lambs had been used in the previous test (trial V). The adults in group VII were given 6 Gm. and those in group VIII were given 8 Gm. The average estimated weight of the adults was 165 lb. Based on the reduction in e.p.g. counts 14 days after treatment, it appeared that good anthelmintic control had been achieved (Table 4, groups V–VIII).

Trial VII.—In August, 1958, at Midland, Mich., 29 lambs were allotted to 6 groups and a special feeding experiment was initiated. During the course of the feeding study, routine weekly individual fecal examinations indicated a substantial rise in e.p.g. counts with a range of 1,514 to 3,580 after 3 weeks. At this time, the lambs were treated with 75 mg./kg. of the organic phosphate as a drench, and individual fecal samples were taken on the 6th, 15th, and 35th day after treatment. Group weights were taken at weekly intervals, and individual weights taken biweekly. Sheep were housed in concrete-floored pens during the experiment. Results of this trial (Table 5 and 6) indicated excellent control of parasites and improvement of weight gains following treatment.

Trial VIII.—This trial was conducted with pastured sheep during August and September of 1958 near Clare, Mich. Pretreatment e.p.g. counts ranged from 524 to 8,028, with an average e.p.g. of 3,602. The principal parasites present were *Haemonchus, Trichostrongylus, Strongyloides, Oesophagostomum,* and *Moniezia.* Evaluation of anthelmintic activity was based on the average of composite pretreatment e.p.g. counts as compared to the average of composite samples taken from each group 15, 30, and 44 days post-treatment (Table 7). The pretreatment average e.p.g. represents the counts obtained in 3 composite samples made up of fecal samples from 30 individual lambs. The post-treatment average e.p.g. represents counts obtained in 2 composite samples made up of fecal samples from 20 individual lambs in each group at 15, 30, and 44 days post-treatment. Examination of the results (Table 7) indicates acceptable control of internal parasites with 50 and 100 mg./kg.

Trial IX.—Tests were initiated in May, 1958, at Midland, Mich., to determine the anthelmintic efficacy of the organic phosphate when fed in the ration. A 10% soybean meal premix was used to prepare the special diets. The anthelmintic efficacy

TABLE 6—Influence of Treatment on Weight Gains of Lambs Reported in Table 5

Group No.	Average weekly gains (lb.)							
	1st week	2nd	3rd*	4th	5th	6th	7th	8th
1	1.8	0.9	0.2	3.3	2.6	2.9	0.8	5.0
2	2.4	2.5	2.7	3.3	2.6	1.2	3.0	4.9
3	3.4	0.8	1.9	3.0	1.8	3.1	3.0	3.7
4	3.5	1.5	2.0	2.6	2.5	2.3	3.4	3.5
5	0.3	2.0	−0.3	4.8	2.1	2.5	3.2	4.8
6	3.9	1.4	1.2	2.6	3.3	1.6	3.7	4.1
Average	2.55	1.51	1.38	3.26	2.48	2.26	2.85	4.33

* Lambs were treated at the end of the 3rd week.

TABLE 7—Comparison of Anthelmintic Effectiveness of the Organic Phosphate and Phenothiazine-Lead Arsenate Mixture in Lambs Maintained on Pasture

Group No.	No. lambs	Treatment	Av. dose (mg./kg.)	Average total e.p.g. counts				Av. post-treatment e.p.g. % change (days post-treatment)		
				Pre-treatment	Days post-treatment					
					15	30	44	15	30	44
1	39	Organic phosphate	25	3,602	2,145	2,062	1,624	−40	−43	−55
2	41	Organic phosphate	50	3,602	774	1,424	892	−79	−60	−75
3	42	Organic phosphate	100	3,602	604	820	772	−83	−77	−79
4	50	Phenothiazine-lead arsenate*	480–19	3,602	1,638	1,300	1,204	−55	−64	−67
5	48	Controls**	.....	3,602	7,776	4,618	1,684	+116	+28	−53

* One lamb died during course of trial from supposed parasitism.
** Five control lambs died during first 4 weeks of trial, all suffering severe *Haemonchus* infections.

was determined by reductions in e.p.g. counts taken on individual sheep before treatment and on the 6th and 7th day following treatment. Twenty lambs were separated into 4 groups of 5 each and treatment administered in the daily grain ration for 1, 3, and 5 days, respectively, to achieve a total anthelmintic dose for each sheep of 100 mg./kg. One group remained as untreated controls. Results are reported (Table 8).

Trial X.—Two crossbred calves weighing 357 and 361 lb., respectively, were maintained on a grain ration in individual stalls at Lake Jackson, Texas, during February and April, 1959. These calves were used to determine the possible effectiveness of the compound when given in the feed for 3 to 5 days. A 10% soybean meal premix was mixed with the daily grain ration.

Calf 773 had an average pretreatment e.p.g. count of 928. The anthelmintic was added to the grain ration, beginning on February 24, for 3 days at a daily rate of 15 mg./kg., giving a total dosage of 45 mg./kg. All feces passed were examined for nematode parasites until the 4th day after initiation of treatment, at which time the elimination of worms had terminated. The average e.p.g. count on the 6th, 7th, and 8th day after termination of treatment was 250 (73% reduction). The following parasites were recovered from the feces after treatment: *Haemonchus placei*, 56; *Trichostrongylus axei*, 160; *Ostertagia ostertagi*, 480; *Cooperia* spp., 7,040.

On March 18 (22 days post-treatment) the calf was treated with 43 mg./kg. of the organic phosphate and 231 mg./kg. of phenothiazine. Again, the worms were collected from the feces after treatment and the following were recovered: *Bunostomum phlebotomum*, 1; *Oesophagostomum radiatum*, 43; *O. ostertagi*, 50; *Cooperia* spp., 50.

On March 23, the e.p.g. count was 2, indicating that the 2nd treatment had removed most of the remaining worms.

Calf 765 was treated in a similar manner. The average pretreatment e.p.g. was 124. The anthelmintic was given in the daily grain ration at a dosage of 10 mg./kg. for 5 days, beginning on April 28, for a total of 50 mg./kg. This treatment reduced the e.p.g. count to 4 and removed the following parasites: *H. placei*, 28; *O. ostertagi*, 300; *Cooperia* spp., 1,700; *Oe. radiatum*, 20.

On May 6 (8 days post-treatment), the calf was treated with 43 mg./kg. of the organic phosphate and 268 mg./kg. of phenothiazine. No worms were passed following this treatment and the e.p.g. count remained at 4, indicating control of the parasites by the feeding of the compound for 5 days at 10 mg./kg. per day.

This trial suggested that removal of *Haemonchus, Ostertagia, Cooperia,* and *Trichostrongylus* could be obtained by incorporation of the organic phosphate in the feed. *Oesophagostomum* was apparently controlled with the 10 mg./kg. dosage for 5 days but not with the 15 mg./kg. for 3 days. Although treatment of 2 animals does not furnish conclusive evidence, the possibilities of a split-level dosage regimen were shown.

TABLE 8—Anthelmintic Efficiency of the Compound as a Feed Additive

No. Sheep	Total mg./kg. in grain supplement	No. days fed	Total e.p.g. % reduction 7 days post-treatment
5	100	1	96
5	100	3	92
5	100	5	95
5	.....	..	−65

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SUMMARIO IN INTERLINGUA

Activitate Anthelminthic de un Phosphato Organic in Bestial e Oves

Le reduction del numeration de ovos helminthic, le retrovation de vermes vacuate in le feces post le tractomento, e le efficacia notate in studios critic indica que 4-tert.-butyl-2-chlorophenol-methyl-methyl-phosphoramidato ha bon proprietates anthelminthic pro parasitos gastrointestinal. Esseva trovate que doses de 40 mg/kg o plus in bestial e de 75 mg/kg o plus in oves provide un bon o excellente repression del parasitos. Le resultatos de limitate essayos, in que le composito esseva miscite in le alimento, indica que le material es appetibile e efficace per iste methodo de application.

Urolithiasis. I. Incidence of Bladder Calculi, Urine Properties, and Urethral Diameters of Feedlot Steers

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SUMMARY

Urinary bladders collected from 491 steers were examined for presence of calculi. The urine was tested to determine pH, surface tension, and specific gravity; the penile urethral diameter was measured for most steers.

The incidence of bladder calculi was 66.4%. Mean pH of urine from steers with calculi (7.74) was significantly different ($P>0.05$) from the mean pH (7.63) of urine from steers without calculi. There was no significant difference between mean surface tension of urine from steers with calculi (71.1) and from those without calculi (71.4). There was no significant difference between mean specific gravity of urine from steers with calculi (1.0209) and from those without calculi (1.0209). The mean urethral diameter of steers with calculi (0.279 inch) was not significantly different from the mean urethral diameter of steers without calculi (0.282 inch).

INTRODUCTION

Although urolithiasis is recognized as a serious problem in ruminants, little information has been published on the properties of urine from animals which have urinary calculi as opposed to the properties of urine from animals which do not have calculi.

Review of Literature

Newsom [6] indicated that deposition of urinary salts depended on reaction (pH) of the urine. He concluded that diet determines urine reaction and is therefore influential in formation of calculi in cattle and sheep. Pontius *et al.*[8] also found that reaction of urine depends on the feed of the animal. They also reported that sheep on alfalfa or beet rations produced basic urine ash; whereas, a cereal grain or bran ration caused the production of neutral urine.

Newsom *et al.*[7] concluded that sheep fed cane fodder, bran, and white corn ration had more calculi than those given alfalfa or beet tops. They also found that with more calculi, total urine output was low, pH was more nearly neutral, and specific gravity and total solids were low. Swingle and Marsh [12] reported that calves with a limited water intake had decreased total urine volume, increased specific gravity, and slightly higher pH. Comparing urine normal and vitamin A-deficient steers, Swingle and Marsh [13] found no consistent difference between specific gravity, pH, or surface tension determinations.

Elam *et al.*[2] found that urine from steers fed alfalfa had a smaller concentration of total solids and was significantly more alkaline than urine from steers fed wheat straw. Lindley *et al.*[4] found there was no apparent association between occurrence of calculi and differences in urine pH in sheep. Johnson *et al.*[3] showed that pH of urine does not predispose wether lambs to calculi formation.

According to Butt,[1] hydrogen-ion concentration of urine from human beings has significant importance as it is related to the precipitability of urinary colloids. He stated that as pH of urine increases, urinary colloids are more liable to rapid precipitation. In man, Butt also found that surface tension of urine from individuals who formed kidney calculi was noticeably higher than that of urine from those who did not form calculi.

According to Puntriano,[9] the determination of surface tension provides valuable information on colloidal activity. He stated that increased surface tension indicates a decreased amount of active colloidal particles, and vice versa. Puntrano concluded that surface tension determination is important in studying the concentration of protective colloids and the tendency toward kidney stone formation.

Smiddy [10] found that the only correlation between surface tension and other factors was that changes in surface tension were inversely proportional to specific gravity.

Marsh and Safford [5] observed a difference in the urethral diameters of steer calves castrated at different ages and bulls. They concluded that these differences could be a factor in the lower incidence

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of clinical urolithiasis observed in bulls and in steers castrated at 6 months of age as compared to steers castrated at 2 weeks to 2 months of age.

Swingle [11] stated that it is believed occluding calculi are less frequent in bulls than in steers. He theorized this could possibly be explained by differences in dimensions of the urethra or in the greater force of urination in the bull.

This report describes the results of a study on incidence of urinary calculi, urine property determinations (pH, surface tension, and specific gravity), and urethral diameters of feedlot steers. The study was conducted to determine incidence of bladder calculi in steers and to find a correlation between urine properties that would be significantly different between animals which form calculi and those which do not. Such a finding would be a valuable aid in the early diagnosis of clinical urolithiasis and possibly would provide valuable information regarding the cause of urinary calculi.

Materials and Methods

Data reported in this study were obtained from 491 finished steers ranging in age from 1 to 2 years and slaughtered at a local slaughter plant. The animals came from 3 major areas: Magic Valley and Boise Valley of Idaho and Jordon Valley of Oregon. The majority of the steers slaughtered were Herefords; few were Aberdeen Angus or Holstein-Friesian.

At slaughter, each urinary bladder was removed intact, rinsed in cold water, dried with a cloth towel, and opened; the bladder contents were emptied into a black photographic tray.

A pH meter* was used to determine urine pH as soon as possible after the bladder contents had been emptied into the tray. After urine pH was determined, each urine sample was examined against the dark tray for urinary calculi. Calculi, when present, were recovered from the urine specimen and a portion of the urine from each bladder was placed in a specimen bottle for further study.

Urine samples were returned to the laboratory where surface tension and specific gravity determinations were performed. Surface tension readings were measured** and the results expressed in dynes per centimeter. Specific gravity was determined with a urinometer.†

The penis was removed from each of 422 steers. Each penis was transected at the insertion of the retractor penis muscle, and potential urethral diameters were measured and recorded. This measurement was taken with a graduated, cylindrical rod which had been calibrated to read in hundredths of an inch. The rod was inserted into the urethra in the direction of the glans penis. Only enough pressure was applied to realize the widest potential urethral diameter, and in no instance was enough pressure applied to cause the urethral lining to be stretched forcibly or torn.

Results

Of the 491 steers examined for bladder calculi and tested for urine properties, 326 (66.4%) had calculi in the bladder.

The results of determinations made on urine from these steers are summarized (Table 1). The number of samples tested varied due to the fact that all bladders did not contain enough urine to allow a sufficient sample for all tests.

The mean pH of urine from steers with bladder calculi was 7.74; whereas, the pH of urine from steers without calculi was 7.63. This difference in mean pH was statistically significant ($P>0.05$).

Data on the relationship between surface tension of urine from steers having bladder calculi and from those without bladder calculi did not reveal a significant difference. Mean surface tension of urine from those steers with calculi was 71.1; that of urine from steers not forming calculi was 71.4.

The mean specific gravity of urine from steers with bladder calculi and from steers without calculi was 1.0209.

There was no significant difference between mean urethral diameters of steers with bladder calculi (0.279 inch) and of those without bladder calculi (0.282 inch).

TABLE 1—Urine Properties of Steers With and Without Bladder Calculi

Property	Steers with bladder calculi				Steers without bladder calculi			
	No. of steers	Mean	Standard deviation	Range	No. of steers	Mean	Standard deviation	Range
pH	326	7.74	0.51	5.7 to 8.1	165	7.63	0.57	6.2 to 8.8
Surface tension (dynes/cm.)	325	71.10	2.60	64 to 76	162	71.40	2.60	62 to 76
Specific gravity	323	1.0209	0.0100	1.000 to 1.040	160	1.0209	0.0197	1.003 to 1.040
Urethral diameter (inch)	281	0.279	0.026	0.21 to 0.37	141	0.282	0.031	0.21 to 0.38

* Beckman Model N, manufactured by Beckman Instruments, Inc., Fullerton, Calif.

** Revici Urotensiometer, produced by Clay Adams Co., Inc., New York, N.Y.

† Squibb Urinometer, produced by Gomco Manufacturing Co., Buffalo, N.Y.

Discussion

The data of this report concern finished feedlot steers fed in the Caldwell area. Of the 491 steers examined, 326 (66.4%) had calculi in their urinary bladders. In spite of this high incidence, few clinical cases of urinary obstruction are reported in feedlot steers from this area. However, some of the surrounding range areas report considerable incidence of this disease.

The majority of calculi collected from these steers were small and rounded, ranging in size from 1 to 3 mm. in diameter. In only 4 of the 326 steers with bladder calculi were the calculi large enough that they might not pass through the urethra. However, many of the calculi which cause obstruction in steers in the surrounding range areas appear to be aggregates of similar small calculi.

Although there was a significant difference between pH of urine from steers with and without bladder calculi, there was no difference between other properties measured.

Urine determinations may have been more meaningful if kidneys had also been examined for calculi and the relationship between kidney calculi, bladder calculi, and urine properties had been studied.

In this area, the incidence of clinical urolithiasis in cattle maintained under feedlot conditions is generally low. Therefore, it is possible that with reference to predisposition to calculi formation we were investigating a homogeneous population.

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Summario in Interlingua

Urolithiasis. I. Incidentia de Calculos Vesical, Proprietates del Urina, e Diametros Urethral in Tauros in Pastura

Le vesicas urinari colligite ab 491 tauros esseva examinate pro le presentia de calculos. Le urina esseva testate pro determinar le pH, le tension de superficie, e le gravitate specific. Le diametro urethral penian esseva mesurate in le majoritate del casos.

Le incidentia de calculos vesical esseva 66,4%. In le urina de animales con calculos le valor medie pro pH (7,74) differeva significativemente ($P>0,05$) ab le valor medie pro pH (7,63) in le urina de animales sin calculos. Nulle significative differentia existeva inter le valores medie del tension de superficie in le urina ab animales con calculos (71,1) e de illo in le urina ab animales sin calculos (71,4). Nulle significative differentia esseva trovate inter le valores medie del gravitate specific in le urina ab animales con calculos (1,0209) e de illo in le urina ab animales sin calculos (1,0209). Le valor medie del diametros urethral in animales con calculos (0,279 pollices) non differeva significativemente ab illo del diametros urethral in animales sin calculos (0,282 pollices).

Colostral Antibody to Rabies in Cattle Vaccinated with HEP Flury Strain of the Virus

Holman E. Williams, Ph.D., M.Sc., D.V.M., M.R.C.V.S.

SUMMARY

Rabies antibody of colostral origin was found to have been passed, in appreciable quantity, to 8 of 12 calves born of rabies-vaccinated dams, even when the last vaccination had taken place 10 to 13 months previous to parturition. Such antibody, when present, persisted for an average of 12 to 14 weeks after birth.

The response to rabies vaccination in calves with measurable colostral antibody was not demonstrable by the serologic method used, whereas neutralizing antibody rose markedly when calves free from measurable colostral antibody were vaccinated. Such vaccinal antibody persisted for about 6 months.

INTRODUCTION

Rabies, transmitted by hematophagous bats, is enzootic on the island of Trinidad, and anti-rabies vaccination of cattle has been practiced as a control measure for a number of years. Since 1955, the high egg passage (HEP) Flury strain of the virus has been used exclusively for annual vaccination; previously, inactivated tissue vaccines had been employed. The proper age at which to vaccinate calves of vaccinated dams has not been determined. The following is an account of a serologic investigation into this problem.

Materials and Methods

Virus Strains.—Commercially produced HEP Flury strain of rabies virus of North American origin was used in vaccinating the cattle.

A standard challenge virus (CVS) of rabies fixed in mice, supplied by the National Institutes of Health, United States Public Health Service, was used in the neutralization test. The primary pool was stored at −65 C. and the working pool at −23 C.

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Serum.—Serum was obtained from blood drawn into open bottles and was stored at −23 C. following heat-inactivation at 56 C. for 30 minutes.

Serum-Neutralization Test.—Equal parts of undiluted test serum and dilutions of rabies CVS were mixed. After mixtures had been incubated at 37 C. for 90 minutes, 5 white mice, 3 to 5 weeks of age, were inoculated intracerebrally with 0.03-ml. amounts of the serum-virus mixture per dilution. The titer of the virus in each test was determined in the presence of undiluted cattle serum taken from an unvaccinated animal which, like its unvaccinated dam, was born and raised on Tobago where rabies is not known. Results were calculated by the Reed and Muench [2] method after an observation period of 21 days.

Animals.—Swiss albino mice (Rockefeller strain) were used in the serum-neutralization test.

A mixed herd of government-owned cattle was used. Herd vaccinations were carried out in October, 1954; August, 1956; December, 1957; and March, 1959. Presumably, all adult animals were vaccinated on these occasions. The vaccinal history of the herd prior to 1954 is not on record; however, several of the cattle were imported in 1952 from Jamaica, W.I., where rabies is not known to exist and where anti-rabies vaccination is not practiced. Rabies has never been seen in this herd, but animals on pasture are occasionally bitten by hematophagous bats at night. Calves, from birth to at least 1 month of age, are kept indoors at night in stalls lighted to discourage bats.

Experimental Procedure and Results

Parental Transmission of Rabies Antibody and Its Persistence.—Blood samples were obtained at various intervals during the first 20 weeks of life from 12 calves born in the herd between September, 1958, and January, 1959. The protective properties against rabies of serum specimens obtained between the 1st and 4th weeks of age are shown (Table 1).

Nine of the calves possessed sufficient antibody to protect mice against approximately 100 mouse LD_{50} of virus and, among these, the serum of 2 calves protected against approximately 680 mouse LD_{50} of virus. Of the remaining 3 calves the serum of one (321) protected against approximately 30 mouse LD_{50} of virus, the serum

TABLE 1—The Protective Property Against Rabies of Serum Taken from Calves, 1 to 4 Weeks of Age, Born of Cows Vaccinated with HEP Flury Strain of the Virus

Calf No.	Age (wk.)	Period (to the nearest week) between vaccination of dam and calving	Protective property of undiluted serum given intracerebrally to mice			Dam neutralization index at birth of calf
			Approximately 30 mouse LD_{50}	Approximately 100 mouse LD_{50}	Approximately 680 mouse LD_{50}	
1	2	57	0/5*	.....	1/5	1,318 or more
2	1	57	0/5	.....	0/5	1,905 or more
304	1	43	.....	1/5	.....	1,445 or more
305	1	43	.....	1/5	.....	177
307	2	44	.....	5/5	.....	28
308	2	45	.....	0/5	.....	.....
313	1	45	.....	0/5	.....	457 or more
318	4	54	.....	1/5	.....	89
319	4	54	.....	0/5	.....	1,905 or more
320	4	54	.....	0/5	.....	1,738 or more
321	4	55	2/5	.....	5/5	.....
322	4	55	5/5	.....	5/5	.....

* Numerator = No. of mice dying from rabies; denominator = No. of mice inoculated; .... = not done.

of one (322) failed to protect against this amount of virus, and the serum of one (307) was only tested against approximately 100 mouse LD_{50} of virus. All of the dams of these calves had been vaccinated approximately 10 to 13 months before the birth of the calves, 7 having been vaccinated 12 or more months before. In contrast, the serum of 4 unvaccinated colostrum-fed calves, within the same age range and born in Tobago of unvaccinated Tobago dams, had neutralization indexes of 3 or less.

That the Trinidad calves were not born with rabies antibody but acquired it after birth is shown (Table 2). Blood samples were obtained from 4 of the calves, (2, 305, 313, and 320) at birth, prior to nursing for the 1st time; at 1 or 4 weeks; and at 20 weeks. Blood samples were obtained from their dams at parturition. All 4 calves were born with negligible rabies antibody levels (neutralization indexes between 1 and 2); however, at parturition, all of the dams had appreciable neutralization indexes, varying from 177 to 1,905 or more. The 3 calves tested at 1 week of age had neutralization indexes between 1,445 and 1,738 or more, and the 4th, tested at 4 weeks of age, had 1,000 or more. Clearly, rabies antibody, at high level, was transferred to the calves in the colostrum. The 4 dams concerned had been vaccinated approximately 10 to 13 months prior to calving. By the 20th week, the neutralization indexes of 3 of these calves had decreased markedly and varied from 3 to 26.

The neutralization indexes of serum of the dams of 9 of the calves (Table I) at parturition were, for the most part, appreciable; 7 of the 9 available for testing were over 100 and 5 of these were from 1,318 to 1,905 or more.

The duration of colostral rabies antibody in the calves was determined by the sequential examination of their serum (Table 3). Calves 321 and 322 were excluded because of the low antibody levels present at 4 weeks of age (Table 1).

Of the 10 calves so examined, only 2 (307 and 318) did not have sufficient serum rabies antibody at the age of 8 weeks to protect mice against approximately 70 mouse LD_{50} of virus. The mean duration of serum antibody capable of protecting mice from approximately 70 to 225 mouse LD_{50} of virus

TABLE 2—The Neutralization Indexes of Serum from 4 Cows at Parturition and from Their Offspring Before and After Nursing for the 1st Time

Cow No.	Neutralization index at parturition	Offspring				
		No.	Neutralization index			
			At birth, prefeeding	1 week	4 weeks	20 weeks
C59	1,905 or more	2	2	1,549 or more	.....	.....
C84	457 or more	313	1	1,445 or more	.....	3
C69	1,738 or more	320	1	.....	1,000 or more	14
C16	177	305	1	1,738 or more	.....	26

.... = not done.

TABLE 3—The Duration of Colostral Rabies Antibody in Calves Born of Cows Vaccinated with HEP Flury Strain of Rabies Virus

Approximate mouse LD_{50}	Calf No.	Protective property of undiluted serum given intracerebrally to mice: Age in weeks 8	12	16	20	Dam neutralization index at birth of calf
225	304	0/5*	0/5	1/5	5/5	1,445 or more
	305	1/4	5/5	5/5	5/5	177
	308	0/5	.....	4/4	5/5	.....
	313	0/5	0/5	5/5	5/5	457 or more
70	1	1/5	0/5	1/5	4/4	1,318 or more
	2	0/5	2/5	4/5	3/5	1,905 or more
	307	5/5	.....	5/5	.....	28
	318	4/5	5/5	1/5	5/5	89
	319	1/5	0/5	4/4	5/5	1,905 or more
	320	0/5	0/5	5/5	5/5	1,738 or more

* Numerator = No. of mice dying from rabies; denominator = No. of mice inoculated; .... = not done.

in the remaining 8 calves (of the original group of 12 calves) was at least 12 weeks from birth.

There was a high correlation between the neutralization index of the dam at parturition and the duration of colostral antibody in the calf. Where the neutralization index was low in the dams (dams 305, 307, and 318), the duration of antibody was shorter than in calves whose dams had higher serum antibody levels. The amount of colostrum ingested by the calf no doubt influences antibody duration as well.

The Effect of Colostral Rabies Antibody on Calf Vaccination with the HEP Flury Strain.—A 2nd group of 12 unvaccinated calves was used to determine whether the presence of colostral antibody counteracts the antigenic capacity of HEP Flury strain vaccine. These calves were selected arbitrarily from the herd described without prior reference to their rabies antibody levels. After initial blood samples were drawn, they were immediately vaccinated as recommended by the manufacturers of the vaccine. Blood samples were obtained at intervals thereafter. The results of the tests on the serum of these calves for rabies neutralizing antibody are shown (Table 4).

In all 5 instances where the calves (57, 60, 278, 286, and 287) possessed negligible amounts of antibody (neutralization index of 13 or less), there was at least a tenfold rise in the serum antibody (neutralization indexes between 50 and 1,000) when measured 6 to 8 weeks following vaccination. Thereafter, the antibody levels tended to decline until at the 26th to 27th week after vaccination the neutralization indexes of 4 calves varied between 25 and 79, but in the 5th calf, the antibody level was negligible. The mean neutralization index 26 to 27 weeks following vaccination was 40.

On the other hand, within the limits of the experiment, a rise in antibody level following vaccination was not demonstrated in the 7 instances where calves (56, 58, 59, 61, 276, 283, and 288) possessed appreciable quantities of colostrum-conferred antibody (neutralization indexes between 33 and 7,940). By the 6th to 12th week postvaccination, the neutralization indexes of 4 of these calves became negligible (3 to 12 or less). In the remaining 3, antibody declined by the 8th week following vaccination; in 2, this decline was to ¼ to 1/10 of the prevaccinal levels. The general pat-

TABLE 4—The Response to HEP Flury Strain Rabies Vaccine, in Terms of Neutralizing Antibody, in Calves Born of Cows Similarly Vaccinated

Calf No.	Age, to the nearest week	Neutralization index: Immediately prior to vaccination	Weeks after vaccination: 6	8	12	26–27
288	11	7,940	997	.....	12 or less	9 or less
287	12	9 or less	997	.....	2,138	79
276	20	54	12	.....	3	5
286	14	4	54	.....	44	25
283	16	33	33	.....	5	2
278	20	1	331 or more	.....	57	57
56	7	97	.....	45	....	13
57	7	10	.....	213 or more	....	10
58	6	851 or more	.....	4	....	2
59	4	851 or more	.....	208	....	2
60	4	13	.....	512 or more	....	34
61	4	741 or more	.....	74	....	7

.... = not done.

tern of antibody decline did not differ markedly from that recorded in the unvaccinated calves in the previous investigation. In the 2nd group of calves, at least 7 had colostral antibody; the possibility exists that others, by virtue of their age, may have already lost theirs before the investigation had begun. The mean duration of colostral antibody (neutralization index of 33 or more) was, as could best be determined from the sampling, at least 14 weeks.

Discussion

It has been stated that there is a fair degree of correlation between serum-neutralizing rabies antibody and protection in cattle.[1,3,4] The investigations reported herein were conducted with the rabies immunizing agent (HEP Flury strain) thought to be the best available. No attempt was made to compare vaccines, nor was evidence sought as to the protection, if any, conferred by colostral or vaccinal antibody in the calf. The investigations have, however, shed light on the age at which calves, born of cows vaccinated against rabies, should be given anti-rabies vaccination.

From the evidence presented, it would seem that, when cattle are vaccinated annually, rabies antibody is passed to a high proportion of the offspring and, although the actual protective value of this colostral antibody is unknown, it appears to interfere with the antigenic response to vaccination. There seems to be little to recommend vaccination in such calves before colostral antibody wanes.

In one group of calves, 8 of 12 retained colostral antibody for an average of 12 weeks after birth and, in the other group, 7 of 12 had antibody for an average of 14 weeks. Rather than vaccinate all calves from vaccinated dams at birth, and vaccinate again at about 14 weeks of age, it would seem advisable to withhold rabies vaccination until the 14th to 16th week after birth and then to revaccinate 6 months later when the calves are about 42 weeks or 10 months of age. To vaccinate such calves before colostral antibody wanes may mean that up to 65% become susceptible after they are about 4 months of age.

In these investigations, the antibody in calves responding to vaccination had declined appreciably after a lapse of 6 months. It may be, however, that indeterminable antibody stil affected the response to vaccination and that, in calves from an unvaccinated herd, the duration of measurable vaccinal antibody may be longer lasting.

Although these investigations were carried out on an island where bat-transmitted rabies is enzootic and the cattle in the experiment were not kept in bat-proof surroundings, the possibility of exposure to field rabies and any booster effect having taken place is regarded as remote. Bat bites are not common in this herd and there has never been an evidence of rabies occurring.

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SUMMARIO IN INTERLINGUA

Anticorpore Colostral Anti Rabie in Bestial Vaccinate con le Virus Racia Flury post Multe Passages per Ovos

Esseva constatate que anticorpore anti rabie, de origine colostral, habeva passate in quantitates appreciabile ad in 8 inter 12 vitellos parturite per matres vaccinate contra rabie, mesmo quando le ultime vaccination habeva occurrite inter 10 e 13 menses ante le parturition. Iste anticorpore, quando presente, persisteva durante un periodo medie de inter 12 e 14 septimanas post nato.

Le responsa a vaccination contra rabie in vitellos con mesurabile concentrationes de anticorpore non esseva demonstrabile per le methodo serologic usate. Tamen, le nivello de anticorpore neutralisante montava marcatemente quando vitellos sin mesurabile anticorpore colostral esseva vaccinate. Tal anticorpore vaccinal persisteva durante circa 6 menses.

Agglutinins for 11 Types of Leptospires in Serum from Cattle in Northeastern Texas

Robert M. Pike, Ph.D.; Iain M. Paton, M.R.C.V.S.; Mary Louise Schulze

SUMMARY

Serum specimens from 294 cattle, representing over 100 different herds suspected of having leptospirosis, were tested against 11 leptospiral serotypes. Positive reactions with at least one type at a serum dilution of 1:100 or higher were observed in 84% of the animals. Of 106 asymptomatic cattle from a single herd in which leptospirosis had previously been observed, 87% reacted with at least one of 5 types. Although the majority of serum samples reacted with multiple types, predominant reactions were observed with *Leptospira pomona* in 182 samples, with *Leptospira sejroe* in 150, and *Leptospira grippotyphosa* in 30. Absorption of selected serum samples with *L. pomona* did not remove the agglutinins for either *L. sejroe* or *L. grippotyphosa*.

INTRODUCTION

Most of the leptospirosis in cattle in the United States is believed to be due to *Leptospira pomona*.[15] Infection of cattle with other types has been found in Russia, Israel, Argentina, and elsewhere [1]; and evidence is accumulating that types other than *L. pomona* may be involved in bovine leptospirosis in the United States. The finding of agglutinins for *L. grippotyphosa* in a bovine serum which was negative for *L. pomona*, and several unconfirmed reports of *Leptospira icterohaemorrhagiae* infection in cattle in this area, prompted us to initiate a survey of the occurrence of agglutinins for these and other types in serum specimens from cattle.

Materials and Methods

Specimens of clotted bovine blood were received from 23 veterinarians. In most instances, specimens reached the laboratory within 48 hours after the blood was obtained. The veterinarians were instructed to obtain blood samples either from animals with signs suggestive of leptospirosis or from animals known to have had such signs recently. Over 100 different herds provided 294 specimens. With few exceptions, these herds were located in northeastern Texas. (A statement as to whether or not the animal had been vaccinated with *L. pomona* bacterin accompanied 234 of the specimens; in most instances, the source of the bacterin was not indicated.) Used in addition were specimens obtained for routine brucellosis testing from 106 cattle in a single herd (B). All of the animals in herd B had been given *L. pomona* bacterin. None of them had signs suggestive of leptospirosis at the time the specimens were obtained, but there had been a few serologically confirmed instances of leptospirosis in this herd within the year.

All serum samples were tested by the microscopic agglutination-lysis test. Live cultures of leptospires after 1 to 2 weeks' growth in Korthof's medium were used as antigens. The serum samples from animals with signs were tested against 11 serotypes (Table 2), but only 5 serotypes were used in testing samples from herd B (Table 2). One tenth milliliter of diluted serum was mixed with 0.1 ml. of culture diluted 1:2 or 1:3 to provide about 50 organisms per field, using a 4-mm. objective. After 3 hours at room temperature (23 to 25 C.), a loopful of each mixture was examined under the darkfield microscope, using a 10x objective without a coverslip. Definite agglutination, consisting of clumps of at least 10 to 15 leptospires, was considered a positive reaction. Nonspecific clumping was ruled out by comparison with controls. In a preliminary test, each serum in a final dilution of 1:100 was tested against each of the types. Serum samples positive in this dilution were retested in final dilutions of 1:400, 1:1,600, 1:6,400, and 1:25,600 to determine the titer.

For agglutinin absorption, cultures were killed by the addition of thimerosal* 1:10,000, centrifuged at 10,000 r.p.m. for 30 minutes and washed once with NaCl solution. To the sediment from 15 ml. of culture, 1 ml. of serum diluted 1:50 was added. After incubation at 23 to 25 C. for 3 hours and refrigeration overnight, the mixture was centrifuged at 10,000 r.p.m. for 30 minutes and the supernatant fluid was tested for agglutination.

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* Merthiolate, product of Eli Lilly & Co., Indianapolis, Ind.

TABLE 1—Total Number of Reacting Serum Samples and Frequency of Reactions with Multiple Leptospiral Serotypes

Status	No. of animals	Number of types agglutinated*										Total animals reacting	
		1	2	3	4	5	6	7	8	9	10	No.	%
ANIMALS WITH SIGNS													
Vaccinated**	139	13	27	35	23	10	7	12	5	3	1	136	98
Not vaccinated**	95	16	12	13	5	4	4	1	5	1	2	63	66
Vaccination unknown**	60	7	7	14	6	4	1	4	1	2	2	48	80
Subtotal	294	36	46	62	34	18	12	17	11	6	5	247	84
ANIMALS WITHOUT SIGNS													
Vaccinated†	106	40	35	13	3	0	....	....	....	..	..	91	87
Total	400											338	85

* Agglutination in 1:100 or higher dilution of serum considered positive.
** Tested against 11 serotypes.
† Tested against 5 serotypes.

Results

Table 1 is a summary of the results showing the number of serum samples that gave agglutination in the categories established on the basis of vaccination status and signs. It also shows the frequency with which serum in each category reacted with one or more types of leptospires. Of the vaccinated animals with signs, 98% were positive in contrast to 66% of the animals not given bacterin. Of the 294 animals with signs, including vaccinated and nonvaccinated animals, 84% were positive. Those with no signs at the time the specimens were taken had almost the same frequency of reactors, 87% of 106, even though samples from these animals were tested against 5 types.

Relatively few of the positive serum samples tested against 11 types (36 of 247) agglutinated one type only, and the most frequently observed multiple reaction was the reaction with 3 types. No serum reacted with all 11 types, but 5 serums reacted to some degree with 10 types.

The frequency with which serum in the various categories reacted with each of the 11 types of leptospires is shown (Table 2).

As would be expected, reactions occurred most often with *L. pomona*, regardless of vaccination history or signs. *Leptospira sejroe*, however, agglutinated almost as frequently, even when only those serum samples with titers of 400 or greater were considered. *Leptospira hebdomadis*, *Leptospira ballum*, and *L. grippotyphosa* were also among the more frequently agglutinated types, although most of the *L. ballum* titers were minimal. No serum in a dilution higher than 1:100 agglutinated *Leptospira hyos*.

The data have been analyzed with respect to the type of leptospire producing the strongest reaction. With few excep-

TABLE 2—Distribution of Positive Serum Samples According to Serotype of Leptospira Agglutinated

Leptospira serotype	Seropositive animals with signs						Seropositive animals without signs (91 animals vaccinated)	
	136 animals, vaccinated		63 animals, not vaccinated		48 animals, vaccination unknown			
	% positive	% with titers of 400 or greater	% positive	% with titers of 400 or greater	% positive	% with titers of 400 or greater	% positive	% with titers of 400 or greater
icterohaemorrhagiae	24	4	30	6	23	8	11	0
canicola	24	3	24	6	25	2	12	0
pomona	85	54	59	33	67	38	63	26
grippotyphosa	18	10	11	6	35	17	8	7
hebdomadis	39	20	35	10	31	15	....	....
bataviae	18	3	29	5	23	6	....	....
autumnalis A	11	1	23	5	21	4	....	....
autumnalis AB	29	4	25	6	27	13	....	....
sejroe	64	41	60	29	67	31	61	15
hyos	24	0	19	0	21	0	....	....
ballum	46	4	36	2	50	2	....	....

TABLE 3—Cross Reactions of Serum Samples Reacting Most Strongly or Equally with Leptospira pomona*

Titer for *L. pomona*	Reacted with *L. pomona* only	Reacted with other types	Titer for other types	Type									
				ictero-haemorrhagiae	*canicola*	*grippotyphosa*	*hebdomadis*	*bataviae*	*autumnalis* A	*autumnalis* AB	*sejroe*	*hyos*	*ballum*
100	20	52	100	13	9	4	1	5	2	2	24	3	12
400	9	29	100	8	5	1	3	5	4	6	14	5	11
			400	0	0	2	1	0	0	0	5	0	0
1,600	3	32	100	8	9	4	4	5	9	13	10	11	14
			400	1	0	5	2	1	1	3	1	0	0
			1,600	1	0	1	0	0	0	0	8	0	0
6,400	2	22	100	12	7	1	4	5	7	10	9	5	13
			400	2	1	3	5	2	2	6	3	0	1
			1,600	0	0	0	0	1	0	0	5	0	0
			6,400	0	0	0	0	0	0	0	1	0	0
25,600	0	13	100	5	7	1	4	7	4	1	8	2	6
			400	4	2	0	1	0	1	5	3	0	0
			1,600	1	0	0	1	1	0	3	1	0	0
			6,400	0	0	0	0	2	2	0	1	0	0
			25,600	0	0	0	0	0	1	1	0	0	0
Totals													
No.	34	148		55	40	22	26	34	33	50	93	26	57
%				30	22	12	14	19	18	27	51	14	31

* Of these 182 serum samples, 138 were from animals vaccinated with *L. pomona* bacterin, 26 had not been vaccinated, and in 18 the vaccination history was unknown.

tions, highest titers were observed with 3 types only: *L. pomona, L. sejroe,* and *L. grippotyphosa.* Those serum samples reacting most strongly or equally with each of these 3 types are listed (Tables 3–5); the titers for cross-reacting types are tabulated in relation to the titer of the predominant reaction.

As shown (Table 3), 34 serum samples agglutinated *L. pomona* alone, but only 5

TABLE 4—Cross Reactions of Serum Samples Reacting Most Strongly or Equally with Leptospira sejroe*

Titer for *L. sejroe*	Reacted with *L. sejroe* only	Reacted with other types	Titer for other types	Type									
				ictero-haemorrhagiae	*canicola*	*pomona*	*grippotyphosa*	*hebdomadis*	*bataviae*	*autumnalis* A	*autumnalis* AB	*hyos*	*ballum*
100	19	37	100	3	10	24	2	4	4	0	1	3	10
400	5	24	100	2	4	10	1	11	2	1	1	7	7
			400	0	0	5	3	2	0	0	0	0	0
1,600	2	35	100	5	7	12	5	13	7	3	7	11	13
			400	2	1	8	4	9	0	0	1	0	0
			1,600	1	0	7	0	3	0	0	0	0	0
6,400	2	23	100	8	6	11	0	3	3	3	7	5	11
			400	1	1	4	0	7	4	0	1	0	2
			1,600	0	0	4	1	6	0	1	0	0	0
			6,400	0	0	1	1	2	0	0	0	0	0
25,600	0	3	100	0	0	2	2	0	0	0	1	0	0
			400	0	0	0	0	1	0	0	0	0	0
			1,600	0	0	0	0	1	0	0	0	0	0
			6,400	0	0	1	0	1	0	0	0	0	0
			25,600	0	0	0	0	0	0	0	0	0	0
Totals													
No.	28	122		22	29	89	19	63	20	8	19	26	43
%				15	19	59	13	42	13	5	13	17	29

* Of these 150 serum samples, 105 were from animals vaccinated with *L. pomona* bacterin, 25 had not been vaccinated, and in 20 the vaccination history was unknown.

TABLE 5—Cross Reactions of Serum Samples Reacting Most Strongly or Equally with Leptospira grippotyphosa*

Titer for *L. grippotyphosa*	Reacted with *L. grippotyphosa* only	Reacted with other types	Titer for other types	Type									
				ictero-haemorrhagiae	*canicola*	*pomona*	*hebdomadis*	*bataviae*	*autumnalis* A	*autumnalis* AB	*sejroe*	*hyos*	*ballum*
100	2	8	100	1	2	4	1	1	0	0	2	1	1
400	2	8	100	0	1	5	3	2	1	2	4	1	2
			400	0	0	1	0	0	0	0	2	0	0
1,600	0	8	100	0	1	3	0	0	3	1	3	1	1
			400	0	1	3	1	0	0	0	1	0	1
			1,600	0	0	0	0	0	0	0	0	0	0
6,400	0	2	100	1	1	1	0	2	0	1	0	0	1
			400	0	0	0	0	0	0	0	0	0	0
			1,600	0	0	0	1	0	1	0	0	0	0
			6,400	0	0	0	0	0	0	0	1	0	0
Totals													
No.	4	26		2	6	17	6	5	5	4	13	3	6
%				7	20	57	20	17	17	13	43	10	20

* Of these 30 serum samples, 13 were from animals vaccinated with *L. pomona* bacterin, 6 had not been vaccinated, and in 11 the vaccination history was unknown.

of these had titers above 400. Cross reactions of the remaining 148 samples which agglutinated *L. pomona* most strongly or equally included all of the 11 types. The most frequent cross reaction, however, occurred with *L. sejroe;* 93 of the serum samples in this group agglutinated *L. sejroe.*

Table 4 includes those samples which reacted most strongly with *L. sejroe* or equally with *L. sejroe* and another type. Of these, 28 agglutinated *L. sejroe* only, 9 of them with titers of 400 or greater. It may be worth noting that all 9 animals had been given *L. pomona* bacterin. Two of the serum samples with titers of 25,600 for *L. sejroe* agglutinated *L. pomona* only to a dilution of 1:100. The tendency to react with both *L. pomona* and *L. sejroe* is again reflected in this group, 89 of the samples reacting with *L. pomona. Leptospira hebdomadis* was also frequently agglutinated by samples in this group, with 63 samples agglutinating this type.

The 26 serum samples reacting most strongly or equally with *L. grippotyphosa* are summarized (Table 5). Four agglutinated this type only, but in low titer. Cross reactions in this group occurred most frequently with *L. pomona* and *L. sejroe.*

Representative serum specimens reacting significantly with *L. grippotyphosa* or *L. sejroe* were absorbed to determine whether *L. pomona* would remove the agglutinins for these types (Table 6). Absorption of 2 serum samples (B77 and C99) with *L. pomona* did not significantly reduce the titers for *L. grippotyphosa.* Likewise, absorption of specimens C219 and C187 with *L.*

TABLE 6—Absorption of Serum Samples Reacting with Leptospira grippotyphosa and Leptospira sejroe

Serum	Tested with	Titer			
		Before absorption	After absorption with		
			L. pomona	*L. grippotyphosa*	*L. sejroe*
B77*	*L. pomona*	<100	<100	<100	.....**
	L. grippotyphosa	3,200	1,600	<100	.....
C99*	*L. pomona*	400	<100	400	.....
	L. grippotyphosa	3,200	3,200	<100	.....
C219*	*L. pomona*	400	<100	.....	400
	L. sejroe	6,400	6,400	.....	<100
C187	*L. pomona*	400	<100	.....	400
	L. sejroe	1,600	1,600	.....	<100

* Vaccinated with *L. pomona* bacterin. ** Absorption not done.

pomona failed to remove the agglutinins for *L. sejroe*. In each case, the type used for absorption removed the agglutinins reacting with that type.

Discussion

From these results it would be unwise to draw conclusions as to the national incidence of infection due to various leptospiral serotypes. The bovine populations on which various observations have been made are not necessarily comparable with our own group. The methods of reading serologic tests sometimes vary among investigators. In addition, there is a possibility that previous immunization may have contributed to the reactivity of some of the serum specimens we have tested. As would be expected, our proportion of reacting serum samples is much higher than the 3.5% reported from Ohio, in 1952,[2] and the 12.0% reported from Washington, in 1956,[20] where random samples of bovine serum were examined. Our data are more comparable to the results reported on specimens from animals suspected of having leptospirosis. Thus, in Illinois 19.7 to 24.0% of serum samples on which a test for leptospirosis was requested were positive, [3,8] while in Florida 41.5% of specimens from animals suspected of having leptospirosis were positive.[6]

There can be no question about the predominance of *L. pomona* infection in cattle in this area, since from 59 to 85% of animals in the various categories reacted with this type; from 26 to 54% reacted with titers of 400 or higher. Many of the reactions with other types were undoubtedly the result of infection with *L. pomona*. Morse and Allen [16] reported that 84% of cattle infected with *L. pomona* reacted with *L. icterohaemorrhagiae*, a considerably higher proportion than was found among the cattle we have tested. Cross reactions between *L. pomona* and *Leptospira autumnalis* have also been noticed in bovine serum,[20] in canine serum,[13] and in human serum.[17] However, animals experimentally infected with *L. pomona* did not develop significant reactions with *L sejroe*,[11] even though reactions with *L. icterohaemorrhagiae* and *L. canicola* were common. Hoag [9] did not find leptospiral agglutinins in bovine serum which contained agglutinins for *Brucella* and *Vibrio fetus*. The same author [9] infected calves with *L. sejroe* and *L. ballum*, respectively, and observed that each type elicited homologous agglutinins only.

Although some of the reactions among those samples examined in this study which reacted most strongly or equally with *L. pomona* (Table 3) can be explained on the basis of cross reactions that are known to occur with serum of animals infected with *L. pomona*, it is difficult to account for the reactions with *L. sejroe*. Of these 182 samples, 93 reacted also with *L. sejroe;* 38 of them agglutinated *L. pomona* and *L. sejroe* to the same titer. In addition, 150 serum samples reacted most strongly with *L. sejroe* or equally with *L. sejroe* and another type (Table 4). It is difficult, therefore, to escape the conclusion that many of these animals were probably infected with *L. sejroe* or a closely related type. The possible occurrence of this type in cattle in the United States has been suggested by the observations of others.[6,9,21] The recent isolation of *Leptospira hardjo* from cattle in Louisiana [18] points strongly to the possibility that this related type may be responsible for the appearance of antibodies reacting with *L. sejroe*. Mitchell *et al.*[14] reported a 75% incidence of serologic reactions to *L. sejroe* in a Canadian dairy herd. Atypical mastitis and a slight general malaise were noticed in animals of the infected herd.

Other evidence in these data suggesting that a member of the *L. sejroe* group is involved is the fact that of those samples reacting most strongly or equally with *L. sejroe* (Table 4), 42% also reacted with *L. hebdomadis*, a type known to be related to *L. sejroe*,[18] in contrast to 14% *L. hebdomadis* reactions among those samples reacting most strongly or equally with *L. pomona*. Since *L. hebdomadis* is more closely related to *L. sejroe* than to *L. pomona*, this difference would be expected if *L. sejroe* or a related type were responsible for the antibodies reacting with *L. sejroe*.

Of interest also are the 30 serum samples that reacted most strongly or equally with *L. grippotyphosa*. Reactions with this type were not conspicuous among those samples reacting predominantly with *L. pomona* or with *L. sejroe*, 12% (Table 3) and 13% (Table 4), respectively. Neither has it been shown that serum samples from animals known to be infected with *L. pomona* have any marked tendency to react with *L. grippotyphosa*.[1] Agglutinins for this type, however, have been found in bovine serums

in Illinois[8] and in Florida.[6] Although *L. grippotyphosa* has not been isolated from cattle in the United States, it has been obtained from raccoons[7,12] and foxes[7] and produces a significant cross reaction with *Leptospira australis*.[10] Our observations suggest that *L. grippotyphosa* or a related type may be present in cattle in Texas. This possibility is of particular interest, since 6 out of 31 human cases of leptospirosis, serologically confirmed in this laboratory,[17] have been associated with predominant titers for *L. grippotyphosa*. Some of these have been described in detail.[4,5,19]

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SUMMARIO IN INTERLINGUA

Agglutininas pro Dece-Un Typos de Leptospira in le Sero de Bestial in Texas Nord-Oriental

Specimens de sero ab 294 pecias de bestial, representante plus que 100 differente greges suspecte de haber leptospirosis, esseva testate contra 11 serotypos leptospiral. Reactiones positive con al minus un typo, a dilutiones de sero de 1:100 o plus, esseva observate in 84% del animales. Inter 106 animales asymptomatic ab un sol grege in que leptospirosis habeva previemente essite observate, 87% reageva con al minus un de 5 typos. Ben que le majoritate del specimens de sero reageva con un pluralitate de typos, predominante frequentias de reactiones positive esseva obtenite con *Leptospira pomona* (182 specimens), *L. sejroe* (150), e *L. grippotyphosa* (30). In seligite specimens de sero, absorption con *L. pomona* non eliminava le agglutininas pro *L. sejroe* e non pro *L. grippotyphosa*.

Some Physiologic Data on Goats

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SUMMARY

The normal electrocardiogram, arterial blood pressure (both systolic and diastolic), respiratory frequency, heart rate, spontaneous motor activity, and rectal temperature were studied in goats. The average values for 8 goats were: of systolic arterial blood pressure, 124.9 mm. of Hg; diastolic arterial blood pressure, 97.8 mm. of Hg; respiratory frequency per minute, 28; heart rate per minute, 105; and the spontaneous motor activity for 24 hours, 1.57 miles. The average rectal temperature for 4 goats was 103.4 F. The average values for 7 goats were: P-R interval, 0.13 second; QRS complex, 0.04 second; Q-T interval, 0.32 second; and R wave, 0.06 second.

INTRODUCTION

Little has been reported in veterinary literature on the electrocardiogram, arterial blood pressure, rectal temperature, heart rate, respiratory frequency, and spontaneous motor activity of goats. This study was performed to obtain such data.

Review of Literature

Alfredson and Sykes [1] reported a series of 3 electrocardiographic tracings from each of 97 dairy cows, using the 3 standard limb leads. Platner *et al.*[6] reported electrocardiographic intervals and heart rates in 5 goats, using the 3 standard limb leads. Lank and Kingrey [4] examined a series of 3 electrocardiograms on each of 45 normal lactating dairy cows. Values of intervals were published [7] along with values for arterial blood pressure, heart rate, respiratory frequency, and rectal temperature. These latter physiologic data have also been reported by Dukes.[3] Martin and Beck [5] reported use of a pedometer for recording spontaneous motor activity of horses.

Materials and Methods

All the goats used in this experiment were mature females of mixed breed and various ages. Three of them were pregnant. Their body weights varied from 40 to 110 lb. They were housed in a box stall and were maintained on the same feed and management routine. All were examined for internal parasites and were treated if infected. A permanent carotid loop was established in each goat.[2] A wooden stand with sling was developed for this experiment (Fig. 1). During determinations, the goat was placed on the sling with its legs through the holes of the sling. The forelimbs were tied together, as were the hindlimbs, using separate ropes. The head was secured to the stand (Fig. 1). To record arterial blood pressure and the electro-

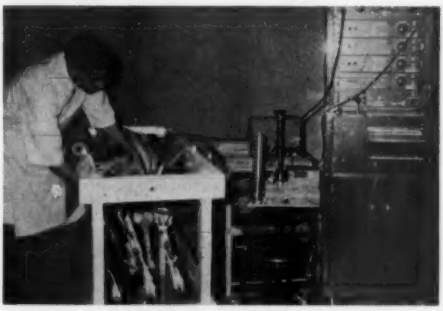

Fig. 1—A multichannel recorder and an electromanometer for recording ECG and arterial blood pressure.

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TABLE 1—Normal Rectal Temperature of Goats

Goat No.	Rectal temperature (F.)
9	103.7
10	103.0
13	103.5
20	103.6
Average	103.4

cardiogram, a multichannel recorder* and an electromanometer* were used (Fig. 1). These were calibrated before each use. Needle electrodes of the ECG were inserted through the skin of the limbs and secured with adhesive tape. A 20-gauge needle was inserted into the lumen of the carotid loop and connected to the manometer (Fig. 2). Using 4 channels, 3 ECG leads and the carotid blood pressure were recorded simultaneously. Respiratory frequency was recorded by means of a stethoscope placed over the nose, and a clinical thermometer was used for recording rectal temperatures.

A pedometer** (Fig. 3) was used to measure spontaneous motor activity of goats housed in a box stall. A thin layer of cotton was applied to the front leg and the pedometer, covered with a second layer of cotton, was secured by adhesive tape. The reading was recorded after 24 hours.

Results

Electrocardiogram.—Seven goats were used for this experiment. The P–R interval, measured in seconds, for all normal goats was found to vary from 0.12 to 0.14 second, with an average of 0.13 second.

TABLE 2—Normal Electrocardiogram Intervals (in Seconds) of 7 Goats

Goat No.	Electrocardiogram intervals in seconds			
	P-R	QRS	QT	R
9	0.12	0.05	0.32	0.07
10	0.12	0.04	0.32	0.05
13	0.12	0.04	0.32	0.04
18	0.14	0.04	0.30	0.08
20	0.14	0.03	0.30	0.06
20A	0.12	0.04	0.31	0.06
24	0.13	0.04	0.32	0.06
Average	0.13	0.04	0.32	0.06

The QRS interval for normal goats ranged from 0.03 to 0.06 second, with an average interval of 0.04 second. The Q–T interval ranged from 0.30 to 0.36 second, with an average of 0.32 second. The P wave was present in all electrocardiograms. The interval of the P wave ranged from 0.04 to 0.08, with an average of 0.06 second.

Arterial Blood Pressure.—Three readings were taken in each goat, and the average of these 3 readings was taken as the blood pressure for that individual. The systolic blood pressure ranged from 111 to 136 mm. of Hg. The mean blood pressure of 8 goats was recorded as 124.0 mm. The diastolic blood pressure ranged from 86 to 113.3 mm., with an average of 97.8 mm.

* Sanborn Company, Waltham, Mass.

** The New Haven Clock and Watch Co., New Haven, Conn.

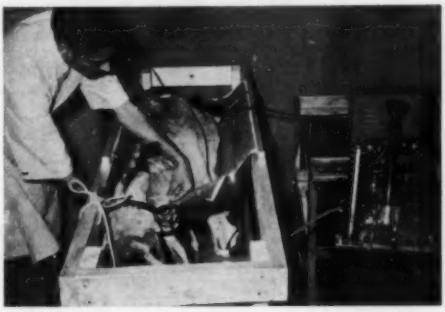

Fig. 2—Inserting a 20-gauge needle into the lumen of the carotid loop for recording arterial blood pressure.

Heart Rate.—The heart rate was recorded as cycles per minute. Three readings were taken in each goat. The average of the 3 readings was taken to be the heart rate of the individual. The individual average values ranged from 90 to 113, with a mean of 105 for 8 goats. In 1 goat, at each reading the heart rate was 90, while in another goat the lowest rate was 100 and the highest was 150.

Rectal Temperature.—The body temperature ranged from 103.0 F. to 103.7 F., with an average of 103.4 F. for 4 goats.

Respiratory Frequency.—Three readings were taken in each animal and the average

Fig. 3—A pedometer for measuring spontaneous motor activity.

TABLE 3—Normal Values for Arterial Blood Pressure, Respiratory Frequency per Minute, Heart Rate per Minute and Spontaneous Motor Activity in Miles for 24 Hours for 8 Goats

Goat No.	Arterial blood pressure (mm. of Hg)		Respiratory frequency/ minute	Heart rate/ minute	Spontaneous motor activity (miles/24 hr.)
	Systolic	Diastolic			
9	136.0	113.3	25	113	3.00
10	134.3	96.6	33	90	0.12
13	117.3	91.0	35	103	0.80
14	125.0	112.0	22	100	1.20
18	131.0	98.0	28	103	1.70
20	111.0	86.5	32	125	2.25
20A	119.6	86.0	21	113	2.00
24	125.0	99.0	30	90	1.50
Average	124.9	97.8	28	105	1.57

was taken to be the rate for the individual goat. Respirations ranged from 21 to 35 per minute, with a mean for 8 goats of 28 per minute.

Spontaneous Motor Activity.—The reading was taken in miles for 24 hours, as recorded by the pedometer. The reading ranged from 0.12 to 3 miles, with an average of 1.57 miles for 8 goats.

Discussion

Electrocardiogram.—Determinations of the P–R interval in this study confirmed the work of previous workers [7] and were in close agreement with some other workers.[6] The QRS, Q–T, and P wave intervals were also in close agreement with other published data. The Q–T interval varied inversely with the heart rate, a shorter interval generally being associated with a faster heart rate and a longer interval with a slower heart rate. The range of values for heart rate was found to be in close agreement with that reported by Spector,[7] but higher than that reported by Dukes.[2]

Arterial Blood Pressure.—The average systolic blood pressure was found to be in close agreement with published data,[7] but the diastolic blood pressure was found to be higher. The mean blcod pressure was found to be in close accord with one published by Dukes.[2]

Rectal Temperature.—The average of the rectal temperatures was found to be in accord with those published by others.[2,7]

Respiratory Frequency.—The range of respiratory frequency was closely in agreement with that reported by Spector *et al.*,[7] but was higher than that published by Dukes.[2]

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SUMMARIO IN INTERLINGUA

Datos Physiologic in Capras

Le valores e characteristicas normal del electrocardiogramma, del tension de sanguine arterial (systolic e diastolic), del frequentias respiratori e cardiac, del spontanee activitate motori, e del temperatura rectal esseva studiate in capras. Le valores medie, a base de observationes in 8 capras, esseva: un systolic tension de sanguine arterial de 124,9 mm de Hg, un diastolic tension de sanguine arterial de 97,8 mm de Hg, un frequentia respiratori de 28 per minuta, un frequentia cardiac de 105. Le spontanee activitate motori amontava a 1,57 millias in 24 horas. Le temperatura medie rectal pro 4 capras esseva 103,4 F. Le valores medie del electrocardiogrammas de 7 capras esseva: un intervallo P–R de 0,13 secundas, un complexo QRS de 0,04 secundas, un intervallo Q–T de 0,32 secundas, e un unda R de 0,06 secundas.

Some Effects of Triflupromazine Hydrochloride on Goats

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SUMMARY AND CONCLUSIONS

Triflupromazine hydrochloride was administered intravenously to goats at 3 dose levels: 0.5, 1.0, and 2.0 mg. per pound of body weight. To determine the effect of the drug on spontaneous motor activity during a 24-hour period, 2 doses of the drug were administered to 6 goats with a 12-hour interval between the 1st and 2nd doses. A single dose of 1 mg./lb. was used in 2 goats.

Depression, labored breathing, cyanosis of mucous membranes, salivation, and incoordination of hind quarters were the signs observed after intravenous administration of the drug to goats. It caused respiratory depression in the majority of goats, though not in all of them. Statistically, the depression was insignificant.

An increased heart rate was observed in the majority of goats at 0.5 and 1.0-mg. levels. Statistically, the difference was insignificant at 0.5 mg./lb. but significant at 1.0 mg./lb.

The drug caused hypotension at all dose levels in the majority of goats, but it was statistically insignificant. The hypotensive effect lasted longer with higher doses.

Some hypothermic effect was seen, but it was statistically insignificant. Depression of spontaneous motor activity was observed in 6 of 8 goats tested with a pedometer. It was statistically significant at the 5% level. No changes that could be definitely attributed to this drug were observed in electrocardiograms.

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The authors are indebted to the Squibb Institute for Medical Research, New Brunswick, N.J., for financial support of this project.

INTRODUCTION

This study was made to determine the effects of a tranquilizer, triflupromazine hydrochloride,* on respiratory frequency, heart rate, arterial blood pressure, rectal temperature, electrocardiogram, spontaneous motor activity, and general clinical signs in goats.

Review of Literature

Triflupromazine hydrochloride is a phenothiazine derivative. Chlorpromazine, which is also a phenothiazine derivative, has been extensively studied. Because triflupromazine hydrochloride is pharmacologically similar to chlorpromazine, some pharmacologic actions of chlorpromazine are reviewed: Duff *et al.*[4] observed that when chlorpromazine was administered intravenously, the heart rate was slightly increased. Dobkin [3] and others reported that pulse rate rose moderately. Pea *et al.*[13] observed that acute hypotension developed in human patients after chlorpromazine was administered, while Azima and Durost [1] noticed a moderate hypotensive effect. Feldman *et al.*[5] reported that rapid intravenous injection of chlorpromazine caused a drop in blood pressure in a mongrel cat. Martin and Beck [10] found that 2 to 4 mg./kg. of chlorpromazine intramuscularly produced hypotension in dogs.

Moyer [11] and others reported that none of the human control subjects developed electrocardiographic changes after chlorpromazine administration. Lank and Kingrey [9] stated that electrocardiograms in tranquilized cows revealed a greater range of interval lengths in heart rate than did those of untreated normal cows.

Two to 4 mg./kg. of chlorpromazine in horses produced a slight fall in rectal temperatures, according to Martin and Beck.[10] Hoerlein and Marsh [6] reported that there was a slight fall of temperature from 1½ to 24 hours after chlorpromazine administration in calves. On the other hand, Owen and Neal [12] found that chlorpromazine caused slight hypothermia in horses.

* Vetame, produced by E. R. Squibb and Sons, New Brunswick, N.J.

Martin and Beck [10] reported 10 trials in which spontaneous motor activity was measured in horses; in 6, there was a decrease in activity during the 24-hour period after chlorpromazine administration, and in 4, the activity increased. Davis [3] found that chlorpromazine in rhesus monkeys produced a marked reduction in all activities when administered in quantities significantly smaller than those required to produce "taming."

Triflupromazine was administered to dogs at a rate of 10 to 80 mg./kg. of body weight, 5 days a week, for 15 or 16 weeks. No significant changes were observed in the electrocardiogram. The immediate effect of the drug on blood pressure was depressant. As a persistent effect, pulse rate decreased in unanesthetized dogs given triflupromazine; there was insignificant change of pulse rate in anesthetized dogs.[14]

Materials and Methods

Readings were taken according to the method previously described by the authors.[7]

Triflupromazine hydrochloride is 10-3(-dimethylamino propyl)-2-(trifluoromethyl) phenothiazine hydrochloride. Its empirical formula is $C_{18}H_{19}F_3N_2S \cdot HCl$.

Reading were taken with 3 dosage levels of 0.5, 1.0, and 2.0 mg./lb. of body weight (Tables 1, 2, 3). With the animals in the stand, a 20-gauge needle was inserted into the lumen of a permanent carotid loop and then connected to a manometer tube.[7] After each reading, the needle was stoppered. The stopper was removed and the needle was flushed and cleaned of clot with heparinized water prior to each subsequent reading. Readings were taken at half-hour intervals.

Results

Clinical Signs Observed After Administration.—At 0.5 mg./lb. of body weight, about 5 minutes after intravenous administration the goats became depressed. There was labored breathing, the mucous membranes became slightly cyanotic, and there was slight dribbling of saliva.

At 1 mg./lb. of body weight, and about 5 minutes after intravenous administration of the drug, the goats became depressed, with eyes half closed, and there was labored, stertorous breathing and slight cyanosis of the mucous membranes. Some frothy salivary secretion was observed.

At 2 mg./lb. of body weight, there was an excessive, frothy, thick, salivary secretion. The mucous membranes were more cyanotic. In the case of goat 14, there was violent movement of the whole body and extreme dyspnea just before death. There was frequent urination and passage of flatus. In almost all goats, incoordination of hind quarters was also observed. In many, there was trembling of the whole body. Goat 9 passed coffee-colored urine about 4 hours after drug administration.

Effects of the Drug on Respiratory Rate.—At 0.5 mg./lb. of body weight, 7 goats, weighing 40 to 110 lb. were used. The mean body weight was 91.9 lb. Definite respiratory depression was observed in 5

TABLE 1—Effects of Triflupromazine Hydrochloride on Respiration Rate, Heart Rate, and Blood Pressure of Goats at a Dose Level of 0.5 mg. per Pound of Body Weight

Goat No.	Respiration per minute			Heart rate per minute			Blood pressure (mm. of Hg)		
	Minutes postinjection			Minutes postinjection			Minutes postinjection		
	30	60	90	30	60	90	30	60	90
9	15	14	....*	90	90	.....*	114** 95†	120 118	.....* .....*
10	30	28	28	110	90	80	110 88	140 105	140 115
13	27	26	26	120	100	100	140 117	146 108	130 102
18	22	24	20	120	100	120	95 88	122 102	106 85
20	38	36	36	160	160	160	107 85	105 86	105 85
20A	15	20	20	110	120	150	112 85	100 84	103 90
24	32	22	30	120	110	100	140 125	138 120	135 119
Mean	25.5	24.3	26.6	118.5	112.8	101.6	116.8 97.5	123.0 101.8	119.8 99.3

* Due to technical difficulty, no recording was made. ** Systolic. † Diastolic.

TABLE 2—Effects of Triflupromazine Hydrochloride on Respiration Rate, Heart Rate and Blood Pressure of Goats at a Dose Level of 1.0 mg. per Pound of Body Weight

Goat No.	Respiration per minute			Heart rate per minute			Blood pressure (mm. of Hg)		
	Minutes postinjection			Minutes postinjection			Minutes postinjection		
	30	60	90	30	60	90	30	60	90
9	16	14	....*	130	160	130	105** 93†	120 100	105 85
10	32	15	....	120	90	.....	135 105	143 108	.....
13	26	32	30	140	110	90	75 63	110 88	115 90
14	14	20	20	90	90	100	115 102	105 92	97 84
18	15	23	25	100	80	120	110 94	135 98	118 99
20	35	35	33	160	110	120	88 77	118 97	110 92
20A	19	21	20	90	150	160	142 133	120 100	110 95
24	16	14	15	110	150	140	98 82	102 90	103 90
Mean	21.6	21.7	23.8	117.5	117.5	122.3	108.5 93.6	119.1 96.6	108.3 90.7

* Due to technical difficulty, no recording was made. ** Systolic. † Diastolic.

goats; stimulation, in 2. In goats 9, 10, 13, 18, and 20A, respiratory depression was observed up to 90 minutes after drug administration. The mean respiratory rate for the group was lower than the mean normal respiratory rate throughout. Statistically, the difference was insignificant at the 5% level.

At 1 mg./lb. of body weight, there were 8 goats, weighing 40 to 110 lb., with a mean body weight of 94.1 lb. In 6 goats (9, 10, 13, 14, 18, and 24), the respiratory rate remained below normal throughout. In goat 20, the respiratory rate remained higher than the normal. The mean normal respiratory rate was below the normal respiratory rate throughout. Statistically, the difference was insignificant at the 5% level.

At 2 mg./lb. of body weight, there were 5 goats, weighing 40 to 110 lb., with a mean weight of 92 lb. In all these animals the respiratory rate was lower than normal, even 420 minutes after drug administration. In goat number 14, the respiratory rate dropped to 7 just prior to death.

Effects of the Drug on Heart Rate.—At 0.5 mg./lb. of body weight, elevation of heart rate was observed in 5 of 7 goats 30 minutes after drug administration. In goat 9, a lowered rate was observed at 30 minutes. In 3 goats (20, 20A, and 24) there was a rise in rate at 60 minutes; in 2 (13 and 18), the rate was lower than normal at 60 minutes. In goat 10, the rate was normal at 60 minutes. In 3 (18, 20, and 20A), there was a higher heart rate at 90 minutes; in 2 (10 and 13), the rate was lower than

TABLE 3—Effects of Triflupromazine Hydrochloride on Respiration Rate, Heart Rate and Blood Pressure of Goats at the Dose Level of 2.0 mg. per Pound of Body Weight

Goat No.	Mean respiration per min.	Mean heart rate per min.	Blood pressure (mm. of Hg)	Mean body temperature (F.)
9	15.6	139.2	121.7*/104.9**	.....†
10	21.3	115.1	102.0 / 79.2	102.5
13	25.0	104.0	139.0 /112.0	102.5
14	8.5	115.0	89.0 / 74.0	.....
20A	17.1	132.3	85.7 / 63.7	101.9

* Systolic. ** Diastolic. † Due to technical difficulty, no recording was made.

normal. The mean heart rate was higher than the mean normal heart rate 30 to 60 minutes after drug administration, and was lower at 90 minutes. Statistically, the change was insignificant at the 5% level.

At 1 mg./lb. of body weight, there was increased heart rate in 5 of 8 goats (9, 10, 13, 20, and 24) 20 minutes after drug administration. In goats 14, 18, and 20A, there was depression in rate at 30 minutes. In 6 goats there was increased heart rate 60 minutes after drug administration; there was no change in goat 10; and, in goat 20, the heart rate was lower. In 4 goats (9, 10, 20A, and 24) there was increased heart rate after 90 minutes, whereas in goats 13 and 20 there was lower heart rate after 90 minutes. The mean heart rate was observed to be higher than the normal throughout. Statistically, the changes were significant at the 5% level.

At 2 mg./lb. of body weight, 5 goats were used and heart rate was recorded in all of them. The mean heart rate after drug administration was slightly lower in 1, but was higher in the remaining 4. The heart rate fluctuated widely in all goats during the postadministration period.

Effects on Rectal Temperature.—At 1 mg./lb. of body weight, readings were taken in 3 goats. There was slight lowering of temperature in all goats 30 minutes after drug administration. There was slight lowering of temperature at 60 minutes in goats 9 and 13. Statistically, changes were insignificant at the 5% level.

At 2 mg./lb. of body weight, rectal temperature was recorded in 3 goats. In 2 (10 and 20A), temperatures lower than normal were recorded even 420 minutes after drug administration. In the 3rd goat (13), lower body temperature was recorded up to 360 minutes after drug administration, at which time recordings ceased. The mean temperature in all 3 remained lower than the mean normal temperature throughout.

Effects on Arterial Blood Pressure.—At 0.5 mg./lb. of body weight, there was decreased systolic blood pressure in 6 of 7 goats, whereas in 1 there was an increase in blood pressure 30 minutes after drug administration. After 60 minutes, there was a decrease in 3, and an increase in 4. At 90 minutes, blood pressure was low in 3 of them, and high in 3. Due to technical difficulties, a 90-minute reading was not made in goat 9. The mean arterial blood pressure was always lower than the normal mean blood pressure. Statistically, the changes were insignificant at the 5% level.

In 6 goats, diastolic pressure was lower at 30 minutes; at 60 and 90 minutes it was higher in 4 and lower in others.

At 1 mg./lb. of body weight, there was lower systolic pressure in 6 of 8 goats after 30 minutes; in 2 there was slight rise in blood pressure after 30 minutes. Four goats had lower blood pressure after 60 minutes, and 4 had a rise in blood pressure. Six of them had lower blood pressure after 90 minutes, and 1 had a rise in blood pressure at 90 minutes. Technical difficulties prevented making a 90-minute reading in goat 10. The mean blood pressures at 30, 60, and 90 minutes were lower than the mean normal blood pressure. Statistically, changes were insignificant at the 5% level.

Diastolic pressure was lower in 6 goats and higher in 2 at 30 minutes. It was lower in 4 and higher in 4 at 60 minutes. At 90 minutes, 3 goats had lower pressure, whereas the others had higher diastolic pressure.

At 2 mg./lb. of body weight, there was lower systolic blood pressure in 4 of 5 goats. In 1 goat (13), blood pressure was lower up to 120 minutes after drug administration, at which time it rose above normal and remained high until recordings ceased at 360 minutes.

Diastolic pressure recordings were variable in the 5 goats given 2 mg./lb.

Effects on Spontaneous Motor Activity.—The 8 goats used in this experiment varied from 40 to 120 lb., with a mean weight of 91.8 lb. (Table 4). Six became less active after drug administration, but 2 became more active. Normal mean distance traveled was 1.57 miles, and mean decrease after drug administration was 0.64 mile, or 40.7%. Statistically, changes were significant at the 5% level.

Effects on Electrocardiogram.—The P wave, P-R, QRS, and Q-T intervals showed a wider range of values in each animal, but the values were within the range of values for the normal goats.[7] Some changes were seen in the amplitudes of wave deflections.

Discussion

When this drug was injected intravenously at the rate of 0.5 mg./lb., respiratory rate was lowered in 71.4% of goats at 30, 60, and 90 minutes. When it was injected at a dose of 1 mg./lb., the respiratory rate was lowered in 75% of the animals throughout observation. The results were

TABLE 4—Effect of Triflupromazine Hydrochloride on Spontaneous Motor Activity of Goats

Goat No.	Intravenous dose (mg./lb. body weight)	Reading* in miles for 24 hours before drug (A)	Reading* in miles for 24 hours after drug (B)	Difference between readings (A and B) in miles	Weight (lb.)
1	2	3.00	1.00	−2.00	100
2	1	0.12	0.82	+0.70	60
3	2	0.80	1.25	+0.45	100
5	2	1.50	0.88	−0.62	120
9	2	1.70	1.25	−0.45	110
10	2	2.25	0.70	−1.55	100
20A	1	2.00	1.31	−0.69	40
23	2	1.20	0.25	−0.95	105

* As recorded with a pedometer, New Haven Clock and Watch Co., New Haven, Conn.

statistically insignificant at the 5% level. At the rate of 2 mg./lb., respiratory rate was lowered in all goats.

When action of the drug on the individual animal was considered, it was observed that in goats 9 and 13 the respiratory rate was lowered at all 3 dosage levels. In goat 10, respiration rate was increased at the 0.5-mg. level, but it was decreased at the 1.0- and 2.0-mg. levels. In goat 14, respiratory rate was lowered at both the 1.0- and 2.0-mg. levels. This goat died 60 minutes after administration of 2.0 mg./lb., perhaps due to respiration failure. In goat 18, respiratory rate increased at the 0.5-mg. level, but was depressed at the 1.0-mg. level. In goat 20A, respiratory rate increased at the 0.5- and 2.0-mg. levels. In goat 24, the respiratory rate was decreased at 0.5 mg/lb. of body weight. Taking these facts into consideration, it was observed in this experiment that the drug caused some respiratory depression in a majority of the goats.

With a 0.5-mg. dose, heart rate was increased in 85.7% of the goats at 30 minutes, and in 42.8% at 60 and 90 minutes. At the 5% level, results were statistically insignificant. At the 1.0-mg. level, heart rate increased in 75% of the animals throughout the test. These results were statistically significant at the 5% level. When 2 mg./lb. were used, a fluctuating pulse rate was recorded. Thus, at 0.5- and 1.0-mg. levels, this drug increased pulse rate in the majority of goats in this experiment.

At the 0.5-mg. level, blood pressure was decreased in 57.1% of the goats at 30 minutes, in 42.7% at 60 minutes, and in 28.5% at 90 minutes. At 1.0 mg./lb., lower blood pressure was recorded in 75% at 30 minutes, in 62.5% at 60 minutes, and in 85.7% at 90 minutes. At the 5% level, results were statistically insignificant. At 2.0 mg./lb., blood pressure was lower in all goats up to 60 minutes, in 30% up to 120 minutes, and in 40% throughout. Thus, the drug caused hypotensive effect at all doses in the majority of cases. This hypotensive effect lasted longer with higher doses of the drug.

With 1 mg./lb., temperature was lowered in 2 of 3 goats at 30 and 60 minutes. Normal temperature was recorded at 90 minutes in all 3 animals. At 2 mg./lb., lower rectal temperature was recorded in all the 3 animals, from 30 to 420 minutes. Thus, the drug had some hypothermic effect on goats. These changes were statistically insignificant at the 5% level.

In 86.6% of the goats, spontaneous motor activity was lowered. In 2 goats, the drug was administered in a single dose rate of 1.0 mg./lb. of body weight. Lowered spontaneous motor activity was recorded in 1 of these 2 goats. In all 8 goats, lowered spontaneous activity was recorded in 75%, and the changes were statistically significant at the 5% level.

For testing the effect of the drug on spontaneous motor activity, the drug was administered to goat 20A at 1.0 mg./lb. of body weight. This goat developed signs of stimulation and behaved in a peculiar fashion. She was found hanging from the hay rack with her head down, and probably would have suffocated if not set free. Due to this stimulation, a second dose was not administered to her. After this incident, owing to the small body weight, only 1.0 mg. of the drug per pound of body weight was used in goat 2.

No consistent changes from normal were found in the electrocardiograms after drug administration.

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SUMMARIO IN INTERLINGUA

Certe Effectos de Hydrochloruro de Triflupromazina in Capras

Hydrochloruro de triflupromazina esseva administrate intravenosemente a capras in un de tres nivellos de dosage: 0,5, 1,0, o 2,0 mg per libra de peso corporee. Pro determinar le effecto del droga super le spontanee activitate motori durante un periodo de 24 horas, 2 doses del droga esseva administrate a 6 capras con un intervallo de 12 horas. In 2 capras un sol dose de 1 mg per libra de peso corporee esseva usate.

Depression, respiration pesante, cyanose del membranas mucose, salivation, e incoordination del extremitates posterior esseva le signos observate post le administration intravenose del droga a capras. Illo causava depression respiratori in le majoritate del capras, ben que non in omnes. Statisticamente le depression non esseva significative.

Un augmento del frequentia cardiac esseva observate in le majoritate del capras post le dosage de 0,5 e de 1,0 mg. Statisticamente le differentia non esseva significative post le dosage de 0,5 mg; illo esseva significative post le dosage de 1,0 mg.

Le droga causava hypotension a omne le nivellos de dosage in le majoritate del capras, sed statisticamente isto non esseva significative. Le effecto hypotensive persisteva plus longemente post le doses plus forte.

Un certe effecto hypothermic esseva notate, sed statisticamente isto non esseva significative. Depression de spontanee activitate motori esseva observate in 6 de 8 capras testate con un pedometro. Isto esseva statisticamente significative al nivello de 5%. Nulle alterationes, definitemente attribuibile a iste droga, esseva observate in le electrocardiogramma.

The Use of Tissue Culture-Propagated Bluetongue Virus for Vaccine Preparation

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SUMMARY

Two American strains of bluetongue virus were adapted to bovine kidney cell cultures. The cytopathic effect produced by the virus in these cell cultures was distinct and could be specifically inhibited by immune serum.

Large quantities of potent antigenic material were prepared in tissue culture for vaccine production.

Twelve lots of experimental freeze-dried live-virus vaccine, composed of 2 modified strains of bluetongue virus, were capable of eliciting a definite antibody response in sheep. The vaccine was used effectively and safely to immunize 48 sheep against bluetongue.

INTRODUCTION

Egg-adapted live-virus vaccine has been used since 1951 for the control of bluetongue in South Africa. This type of vaccine, containing 2 American strains of the virus, is now available commercially in the United States.

Literature relating to propagation of bluetongue virus (BTV) in tissue culture is limited. In 1956, Haig *et al.*[6] reported that 5 strains of BTV produced a characteristic cytopathic effect in sheep kidney cell cultures. The use of tissue culture-propagated virus for complement-fixation antigen was suggested by Shone *et al.*[9] Recently, in an extensive study, Fernandes[4] has shown that BTV can be passed serially in various tissue culture systems and that cytopathologic degeneration can be inhibited or neutralized by immune serum. He suggested that "the preparation of a vaccine from tissue culture-adapted virus seems to merit investigation."

The advantages of tissue culture techniques for viral vaccine production are widely accepted. Therefore, attempts were made to produce in tissue culture an antigenic substance suitable for vaccine preparation.

In this report, observations made in adapting 2 American strains of BTV to growth in bovine kidney cells and the development of a live-virus vaccine are presented.

Materials and Methods

Preparation of Bovine Embryo Kidney Epithelial Monolayer Cultures.—Bovine fetuses, 5 to 8 months in development, were obtained from local abattoirs. Kidneys were removed in an aseptic manner, and brought to the laboratory in sterile containers. In this procedure, a modification of the method of Youngner[11] was employed. Following repeated extractions, single cells and cell clumps were filtered and washed in Earle's balanced salt solution (BSS). Centrifuged, packed cells were resuspended in growth medium to a concentration of 1:200 (v/v). This suspension, containing about 600,000 to 800,000 cells per milliliter, was dispensed in 1.0-ml. quantities into 16 x 150-mm. tubes or in 500-ml. amounts into Povitsky bottles. Material in the rubber-stoppered culture vessels was incubated in a stationary position at 36.5 C. In 5 to 7 days, a confluent sheet of tubular epithelial and fibroblast cells formed on the surface of the glass. Immediately prior to seeding, the nutrient fluid was removed and maintenance medium introduced.

The growth medium consisted of Earle's BSS, 80.0%; lactalbumin hydrolysate in a 5.0% sterile solution, 10.0%; and 10.0% sterile fetal calf serum. The pH was adjusted to 7.4 by addition of sodium bicarbonate solution. One hundred units of penicillin and 100 μg. of streptomycin per milliliter were also added. Maintenance medium was of the same composition, except that the fetal calf serum concentration was reduced to 2.0%. All mediums were filtered through a Seitz S-3 pad shortly before being used.

Virus.—Two immunologically identical American strains of BTV, isolated from sheep at the University of California and designated as strains BT-8 and BT-11, were used in this study. These

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had been suitably attenuated by 78 serial passages in embryonating chicken eggs. Additional stock virus preparations were prepared from pooled tissue culture fluids harvested at the time maximum cell destruction was observed. This material was stored in sealed ampules at −50 C. Occasionally, inoculums were held at 4 C. for short periods.

Titration of Virus.—Serial decimal dilutions were prepared with cold maintenance medium, using a different pipette for each dilution. Two-tenths milliliter of each dilution was inoculated into 4 tissue culture tubes. These cultures were examined daily, and the progress of cytopathic changes was recorded. The test was carried no longer than 7 days. The 50% endpoint of infectivity ($TCID_{50}$) was calculated by the method of Reed and Muench.[8]

Growth Curve of Virus in Tissue Culture.—This was determined according to the method of Fastier.[3] The nutrient fluid was removed from cultures previously prepared in five 8-oz. prescription bottles, the cell sheet was washed, and 20 ml. of fresh medium, containing 200 ID_{50} of BTV, was added to each bottle. To permit attachment of virus to the cells, the bottles were held at 36.5 C. for 2 hours. The fluid was again removed, the cells were washed to remove unabsorbed virus, and 20 ml. of fresh medium was added. Before returning the bottles to the incubator, 0.2 ml. of fluid was withdrawn from each container. Thereafter, samples were taken at different intervals (Fig. 1). Each time, the 5 samples were pooled for virus titer determination.

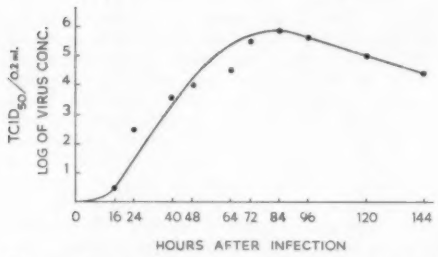

Fig. 1—The growth curve of bluetongue virus in bovine kidney monolayer cultures.

Neutralization Test.—The preliminary qualitative serum-neutralization test was carried out by mixing equal amounts of undiluted serum with an undiluted virus suspension (tissue culture fluid of known titer). All serum samples found to have a complete virus-neutralizing effect were retested to obtain titer endpoint. In the quantitative serum-neutralization test, twofold dilutions of serum were mixed with a constant amount of virus in equal volume. In both tests, the mixtures were incubated at 36.5 C. for 1 hour, then immediately chilled, and 0.2 ml. was implanted into each of 4 tissue culture tubes. The test was concluded when the virus titrated alone produced a complete degeneration of the cell monolayer in the tubes inoculated with 10^{-3} or 10^{-4} dilutions. This regularly was observed on the 5th day following inoculation. All serum samples were inactivated at 56 C. for 30 minutes before use.

Preparation of Vaccine.—The antigenic materials used for vaccine production were harvested from 3- to 4-day-old cultures, when maximum cytopathic changes were evident. Equal parts of each strain were mixed with a suitable virus stabilizing fluid, according to the calculated $TCID_{50}$ titer, and the mixture dispensed into final containers for desiccation under vacuum. Twelve lots of trial vaccine were completed and tested for sterility, safety, and potency.

Testing of the Vaccine.—The minimum field immunizing dose of chicken embryo vaccine is fixed at not less than 2,500 ELD_{50} (1,250 ELD_{50} of each isolate) in 2 ml. To establish a minimum sheep-immunizing dose of tissue culture origin vaccine, virus content in all lots of vaccine was titrated in tissue culture and in embryonating eggs. Three groups of 4 sheep each were vaccinated, using the recommended field dose of 2 ml. from 3 lots of chicken embryo origin vaccine. Twelve similar groups of sheep were inoculated subcutaneously with 2-ml. doses of restored tissue culture origin vaccine of known titer. At the time of vaccination and again 21 days later, serum samples were collected from each animal for serum-neutralization tests. Physical examinations of the sheep were made daily. Morning temperatures only were taken for 5 days. From the 6th through the 11th day, both morning and afternoon temperatures were taken. The last 5 days of the test period only morning temperatures were taken. Observations were concluded on the 16th day, when the temperatures of all sheep returned to normal.

Chicken Embryos.—Fertile White Leghorn eggs, preincubated for 8 to 9 days, were inoculated with 0.2 ml. of BTV via the yolk sac route, applying Gorham's[5] technique. Six eggs were used for each dilution of virus. After inoculation, the eggs were incubated overnight at 35.0 C. in a forced-draught egg incubator. Thereafter, the incubator temperature was reduced to 33.5 C. Death of embryos during the first 40 hours after inoculation was considered due to trauma, and these eggs were discarded. Observation continued for 6 days or until all embryos had died. The embryo lethal dose (ELD_{50}) titer of the virus was calculated according to the method of Reed and Muench.[8]

Suckling Mice.—Litters of 6 or more suckling mice, 2 to 4 days old, were inoculated intracerebrally with either a 20% suspension of BTV-infected mouse brain tissue or with dilutions of tissue culture fluids containing BTV. These infant mice were examined daily, and sickness or death of the animals was recorded. Brains of the sick mice were removed and minced in a Ten Broeck grinder; a 20% tissue suspension was prepared in cold phosphate-buffered saline solution containing 0.5%

bovine albumin fraction V., according to the method of Taylor.[10] Brain suspensions so prepared were centrifuged, dispensed into ampules, and sealed. This material was then shell-frozen by rotating the ampules in a CO_2-alcohol mixture for storage at −50 C.

Sheep.—White-faced lambs, 6 to 8 months of age, were used. Serum collected from each animal was examined for presence of virus neutralizing antibodies. Animals were held in isolation during this period.

Immune Serums.—Antiserums were prepared in mice immunized by repeated intraperitoneal injections of suspensions of infected mouse brain or tissue culture fluids containing BTV, according to the method of Van den Ende *et al.*[2]

Apparatus.—Desiccation was accomplished in a 3-stage refrigeration type freeze-dryer apparatus.*

Results

Cultivation of Egg-Adapted Strains in Embryonating Chicken Eggs.—A number of passages in eggs were conducted to demonstrate the presence of BTV in dead embryos. It was observed that embryos dead of bluetongue infection had a characteristic "cherry red" color when compared to controls which retained a normal pale appearance. The infectivity of the virus in chicken embryos reached a maximum level of $10^{5.2}$ to $10^{5.5}$ ELD_{50} in the 5th consecutive egg passage. This titer was obtained with each strain of virus. Thereafter, using both strains of virus, 4 separate batches of experimental vaccine were prepared, each at different passage levels. These batches were subsequently freeze-dried. Virus titrations of the desiccated vaccines are summarized (Table 1).

Adaptation of Bluetongue Virus to Suckling Mice.—Suckling mice, inoculated intracerebrally with 0.03 ml. of a 1:100 dilution of stock virus (50% chicken embryo suspension), survived the first passages without signs of illness. After 3 or more blind passages, however, all of the mice sickened on the 5th or 6th day postinoculation (PI) and died on the same day, or on the day following appearance of sickness. The incubation period shortened with each passage and, after the 8th or 9th consecutive passage, both strains regularly caused death of infant mice within 2 to 3 days after inoculation.

In bovine kidney cell cultures, inoculated with a tenfold dilution of suspensions of infected mouse brain, the BTV produced characteristic cytopathic changes after 4 to 5 days of incubation. Suckling mice inoculated with these cultures died 3 to 4 days PI.

Propagation of Bluetongue Virus in Bovine Kidney Cells.—Tenfold dilutions of a 50% suspension of chicken embryos, prepared in maintenance medium, were inoculated into bovine kidney cell cultures. In the 1st passage, small foci of degeneration appeared along the margins of the cell sheets on the 4th or 5th day PI. After 3 or more serial passages, focal lesions become scattered throughout the cell sheets within 48 to 56 hours PI. The cytopathic effect of the BTV was characterized by shrinkage of cells and increased granularity. The rapidity and intensity of degeneration increased upon continued passages and a complete (4^+) cell destruction occurred on the 4th or 5th day, depending upon the quantity of virus inoculated. Both strains produced identical cytopathic changes.

Growth Curve of Virus in Tissue Culture.—The growth curve of BTV in bovine kidney monolayer cultures is shown (Fig. 1). In the 1st sample, no virus could be detected. In the 2nd sample, obtained 16 hours PI, the quantity of extracellular virus reached a concentration of $10^{0.5}$. Titer increased rapidly thereafter, reaching a maximum level approximately 84 hours PI. This level remained unchanged for 2 to 3 days before declining.

Testing of the Tissue Culture-Origin Vaccine.—At least 2 vials from each lot of vacuum-dried vaccine were reconstituted and the virus content titrated in tissue culture and in embryonating chicken eggs. Four sheep were vaccinated, using 2.0 ml. of vaccine from these same lots. Results of the virus titration are shown (Table 2).

Some of the 60 sheep vaccinated, either with chicken embryo-origin or with tissue culture-origin vaccines, had a moderate elevation of temperature between the 6th and

* Stokes Model No. 2005-FFFX4, manufactured by F. J. Stokes Machine Co., Philadelphia, Pa.

TABLE 1—Virus Concentration of Vacuum-Dried Chicken Embryo Origin Bluetongue Viral Vaccines

Egg passage level 78+...	ELD_{50}*		Vaccine		
	BT-8	BT-11	Lot No.	ELD_{50}*	Per immunizing dose ELD_{50}
1	3.5	4.0	.....	2.5	3.1×10^2
2	4.0	4.0	101	3.3	1.9×10^3
3	5.0	5.0	102	3.2	1.5×10^3
4	5.0	5.0	103	4.0	1.0×10^5
5	5.2	5.5	104	3.5	3.1×10^4

* Log of reciprocal dilution per 0.2 ml. of inoculum.

10th days postadministration. However, no clinical signs of illness were observed. No BTV antibodies could be demonstrated in any of the 60 serums collected prior to passages in a combination of mouse brain suspension and bovine kidney cell cultures. Van den Ende *et al.*[2] previously reported that infant mice are susceptible to BTV infection; Cabasso *et al.*[1] successfully propagated several strains in suckling hamsters.

TABLE 2—Virus Concentration of Vacuum-Dried Tissue Culture Origin Bluetongue Viral Vaccines Titrated in Tissue Culture and in Embryonating Chicken Eggs

Tissue culture passage level	$TCID_{50}$*		Vaccine			
	BT-8	BT-11	Lot No.	$TCID_{50}$*	ELD_{50}*	Per immunizing dose $TCID_{50}$
8	4.5	5.5	501	2.6	2.3	3.9×10^3
			502	2.3	2.6	1.9×10^3
9	3.5	5.0	503	3.5	2.5	3.1×10^4
			504	3.3	2.6	1.9×10^4
17	5.5	5.5	505	4.5	3.0	3.1×10^5
			506	4.5	3.0	3.1×10^5
			507	4.5	3.0	3.1×10^5
			508	4.5	3.0	3.1×10^5
			510	4.7	3.0	5.0×10^5
			511	4.5	3.0	3.1×10^5
			512	4.5	3.0	3.1×10^5
			513	4.5	3.5	3.1×10^5

* Log of reciprocal dilution per 0.2 ml. of inoculum.

vaccination by serum-neutralization tests in tissue culture. Employing postvaccination serums, complete neutralization was found in the qualitative test, and when twofold dilutions of serum were mixed with a constant amount of virus, a clearly defined titer endpoint, varying from 1:8 to 1:64, was obtained.

Discussion

The application of tissue culture techniques for viral vaccine production has many advantages. In the data presented, it has been demonstrated that BTV can be grown in monolayers of bovine kidney cells with characteristic cytopathic effect, permitting an accurate measurement of virus concentration.

Serial sampling technique was employed for ascertaining the growth curve of virus. This procedure was used by Fastier[3] to determine the growth curve of infectious canine hepatitis virus in tissue culture. The method is simple, easy to perform, and gives reproducible results. Our observation provided an indication of the time that BTV should be harvested for vaccine production. There was no difficulty propagating BTV in test tubes or in producing larger quantities of virus at reasonable cost in Povitsky bottles.

Infectivity of the virus was confirmed by adapting both strains to suckling mice. Different virus lines were obtained by serial

The immune status of animals, vaccinated either with chicken embryo-origin or tissue culture-origin vaccines, could be determined by serum-neutralization in tissue culture. Thus, a serologic test as adjuvant to virus titer determination provided more accurate evaluation of vaccine potency.

Vaccines prepared from tissue culture-adapted virus have proved to be a source of satisfactory antigen. All serum collected 21 days postvaccination neutralized BTV in a significant titer.

Further investigations should be made to determine the nature of cell changes produced by the virus in tissue culture. The primary cell culture, which is a mixed population, may contain some cells that are resistant to the virus. Haig *et al.*[6] observed that fibroblast-like cells survived longer than the epithelial-like cells. Fernandes[4] has found that susceptibility to BTV varied considerably in 10 cell lines studied, and that some were not affected morphologically by the virus.

Inoculations of pure cell lines instead of primary cell cultures, as suggested by Madin,[7] may solve the problem of determining which type of cell is more susceptible and will better support the growth of BTV. The use of pure cell lines may provide a richer virus yield, and a more potent vaccine could conceivably be the result.

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SUMMARIO IN INTERLINGUA

Le Uso de Histoculturalmente Propagate Virus de Lingua Blau in le Preparation de Vaccino

Duo racias american de virus de lingua blau esseva adaptate a culturas de bovin cellulas renal. Le effecto cytopathic producite per le virus in tal cytoculturas esseva nette e poteva esser inhibite specificamente per sero immun.

Grande quantitates de potente material antigenic esseva preparate in cytoculturas pro le production de vaccino.

Dece-duo lots de vaccino experimental de cryo-siccate virus vive, componite de 2 modificate racias de virus de lingua blau, esseva capace a evocar un definite responsa anticorporee in oves. Le vaccino esseva usate in le efficace e salve immunisation de 48 oves contra lingua blau.

The Production of High Concentrations of Foot-and-Mouth Disease Virus in Cultures of Cells on Glass

Richard E. Patty, M.S., Ph.D., and Hilda J. May, B.A.

SUMMARY

A study was made of the effect of concentration, thermal inactivation, redistribution of inoculums, and use of multiple inoculums on the attachment of foot-and-mouth disease virus, type A, strain 119 (FMDV-A119) to cells grown on glass. The most rapid infection of cells occurred when small inoculums of high infectivity were added directly to the cell surface and redistributed every 5 minutes during the attachment period.

The methods used for rapid production of high concentrations of FMDV-A119 resulted in concentrations in the cell portion of 10^{10} plaque-forming units (PFU) per milliliter within 6 hours and concentrations in the fluid portion of $10^{9.2}$ PFU per milliliter within 9 hours after adding virus to the cells.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) has been grown *in vitro* by several methods. Hecke [3] and Frenkel [2] produced virus from tissue fragments suspended in a sustaining medium. Bachrach *et al.*[1] grew the virus in cells cultured on glass. Growth of FMDV in suspended cells first cultured on glass was reported by Patty *et al.*[4]

The use of cells cultured on glass for production of FMDV in high concentration for antigen studies and for physical and chemical characterization is being explored in this laboratory. This report concerns (1) attachment of FMDV to cells under different conditions; (2) accumulation of virus in the cell and fluid portions of cultures; and (3) methods for rapid production of high concentrations of virus in cell cultures.

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Acknowledgment is made to Mr. Robert Goldsmith and Mr. Roger Williams for their valuable technical assistance.

Materials and Methods

Virus.—The FMDV-A119 used was serially passaged 100 times in outgrowths of bovine kidney cells. Harvested infectious fluids were adjusted to pH 7.5 and stored in ampules at −40 C. Before use, the virus was thawed, clarified at 1,500 r.p.m. (380 g) to remove cellular debris, diluted 1:1 in growth medium containing 60% bovine serum (final concentration of serum, 30%), and warmed to 25 C.

Cultures.—Confluent sheets of cells were produced in 5-liter Povitzky bottles and 4-oz. prescription bottles from trypsin-dispersed bovine kidney cells. The Povitzky bottle cultures were used to produce virus and the prescription bottle cultures were used for bioassay. Medium for cell growth was composed of Hanks' balanced salt solution, 2% bovine serum, and 0.5% lactalbumin hydrolysate. Penicillin G, dihydrostreptomycin sulfate, and phenol red were included in concentrations of 100 units per milliliter, 100 μg. per milliliter, and 0.005%, respectively. Cell numbers were estimated from nuclei counts of cells treated with 0.1M citric acid and crystal violet.[5] Unless otherwise stated, each Povitzky bottle culture contained approximately 50 million cells.

Assay Method.—The plaque method described by Bachrach *et al.*[1] was modified for virus infectivity measurements. Duplicate 0.1-ml. volumes of decimally diluted virus solutions were plated on prescription bottle cultures. Originally and after 45 minutes' incubation at 37 C., the inoculum was distributed over the surface of the cells by tilting each bottle. After another 45 minutes, overlay was added, consisting of Hanks' balanced salt solution, 10% bovine serum, 1.5% agar, and 1:30,000 neutral red. Thirty minutes later, the cultures were inverted and incubated at 37 C. for 72 hours before plaques were counted and recorded as plaque-forming units (PFU) per milliliter.

Experiments and Results

Experiment 1.—The efficiency of viral attachment to cells when the virus was added to the fluid portion of the culture was determined. Five milliliters of FMDV-A119 was added and mixed with the fluid portion (245 ml.) of each of 2 Povitzky bottle cultures. One culture received 2.7×10^7 PFU and the other, 2.7×10^5 PFU. Cul-

tures were incubated at 37 C. and samples were removed periodically and assayed for infectivity.

Results (Fig. 1) show the loss of infectivity in the fluids after allowing for thermal inactivation of the virus. Loss due to thermal inactivation during the first 3 hours for each of the concentrations of virus amounted to approximately 50%. During the first 2 hours of incubation, approximately 100 times as much virus was attached to the cells in the culture with the greater concentration of virus. This greater attachment of virus was reflected in early liberation of greater amounts of virus from the cells into the fluid (Fig. 2).

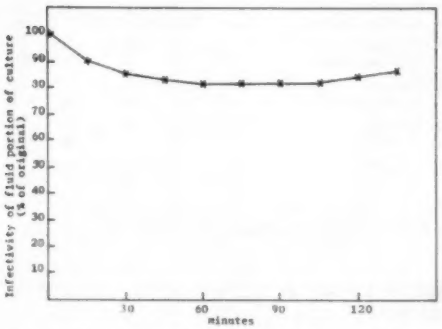

Fig. 1—Loss of infectious foot-and-mouth disease virus, type A, strain 119, from the fluid portion of a culture at 37 C. because of attachment of virus to the cells. Five milliliter of virus inoculum was added to 245 ml. of medium which covered approximately 50 million cells.

Virus in the fluid portion of each culture at peak yield was approximately 2×10^{10} PFU, or a concentration of 8×10^{7} PFU per milliliter (Fig. 2), making the average yield per cell about 400 PFU. The peak titer was reached 4 hours earlier (16 hours), using the higher concentration.

Experiment 2.—The purpose was to estimate the increase in efficiency of attachment known to occur [1] when the virus inoculum is placed directly on the cell layers. Two Povitzky bottle cultures were each inoculated with 2.7×10^{7} PFU of FMDV-A119 (in a 5-ml. inoculum). The 5-ml. virus inoculum was added to the fluid portion of the first culture, as in experiment 1. Fluid was aspirated from the second culture, and the 5-ml. inoculum was distributed over the surface of the cells by tilting the bottle.

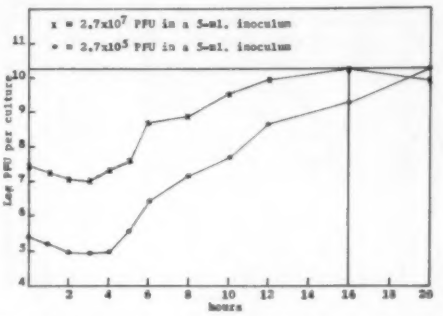

Fig. 2—Loss of infectious foot-and-mouth disease virus, type A, strain 119, from the fluid portion of cultures at 37 C. because of attachment of virus and thermal inactivation and the accumulation of virus in the fluid portion of each culture. Five milliliters of virus inoculum was added to 245 ml. of medium which covered approximately 50 million cells in each culture.

Both cultures were then incubated at 37 C. In the second culture, the 5-ml. inoculum was redistributed over the cell surface every 5 minutes during the first 45 minutes of incubation, after which 245 ml. of growth medium was added. Samples were removed from each culture periodically and tested for virus infectivity.

The increase in efficiency of attachment when the virus inoculum was placed directly on the cell layer is illustrated (Fig. 3). During the first 2 hours after viral addition, approximately 80% of the virus

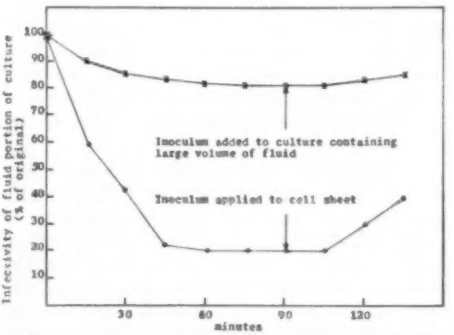

Fig. 3—Loss of infectious foot-and-mouth disease virus, type A, strain 119, from the fluid portion of cultures at 37 C. because of attachment of virus to cells. Medium was added to the 5-ml. virus inoculum in one culture after 45 minutes' incubation at 37 C. to bring the volume of fluid covering the cells to 250 ml. Each culture contained approximately 50 million cells.

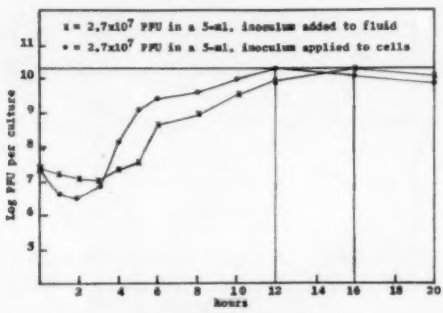

Fig. 4—Loss of infectious foot-and-mouth disease virus, type A, strain 119, from the fluid portion of cultures at 37 C. because of attachment of virus and thermal inactivation and the accumulation of virus in the fluid portion of each culture. Medium was added to the 5-ml. virus inoculum in one culture after 45 minutes' incubation at 37 C. to bring the volume of fluid covering the cells to 250 ml. Virus was liberated into 250 ml. of fluid which covered approximately 50 million cells in each culture.

was removed from the culture fluid where the virus inoculum was added directly to the cells, as compared with 20% where the virus inoculum was added to the fluid portion of the culture. This loss of infectivity was reflected in different rates of release of viral progeny (Fig. 4). The level in the fluid corresponding to the original level was reached 1 hour earlier (3 hours and 10 minutes) in the culture where cells were exposed directly to the 5-ml. virus inoculum.

The yield from the 250-ml. fluid portion of each culture (Fig. 4) amounted to approximately 2×10^{10} PFU, or a concentration of 8×10^7 PFU/ml. The average yield per cell was approximately 400 PFU. The time of peak yield was reduced approximately 4 hours (from 16 hours to 12 hours) by adding the virus inoculum directly to the cell sheet.

Experiment 3.—The effect of redistribution of the virus inoculum on virus attachment to the cells was studied. Appropriately diluted virus in 0.1-ml. aliquots was transferred to 50 4-oz. prescription-bottle cultures after their fluid had been aspirated. The inoculum was distributed over the cell layers by tilting each bottle, and the cultures were incubated at 37 C. for 90 minutes. Ten cultures were left undisturbed during the incubation period. The inoculum was further distributed over the surface of the cells of the remaining cultures in groups of 10 during the 90-minute incubation period as follows: at 45, 30, and 60 minutes; every 15 minutes; and every 5 minutes. After the full 90 minutes of incubation, overlay was added. The number of plaques in cultures of each group was expressed as a percentage of the greatest number. Results of 5 replications are presented (Table 1).

TABLE 1—The Effect on Plaque Counts of the Redistribution of Inoculum Containing Foot-and-Mouth Disease Virus, Type A, Strain 119

Experiment No.	Number of redistributions of virus inoculum				
	0	1	2	5	17
1. Plaques*	8	11	12	12	17
% of maximum	47	65	71	71	100
2. Plaques	10	14	14	15	21
% of maximum	48	67	67	71	100
3. Plaques	20	26	25	27	38
% of maximum	53	69	66	71	100
4. Plaques	21	26	28	31	38
% of maximum	55	68	74	82	100
5. Plaques	43	49	55	63	84
% of maximum	51	58	65	75	100
Mean % of maximum	51	65	69	74	100

* Mean number of plaques in 10 cultures.

The maximal plaque counts occurred when the inoculum was redistributed every 5 minutes before adding overlay. With few exceptions, a decrease in the number of redistributions resulted in a decrease in the number of plaques. The least number of plaques, half of that of the maximal number, occurred in cultures where the inoculum had not been redistributed.

Experiment 4.—The fluid portion of the cultures was reduced to allow virus to accumulate to higher concentrations. Fluid was aspirated from Povitzky bottle cultures, each of which contained approximately 100 million cells. A 5-ml. inoculum of virus was added to each culture and distributed over the surface of the cells, with redistributions being made every 5 minutes for the first 45 minutes. After this 45-minute period at 37 C., 5 ml. of growth medium containing 30% bovine serum was added. Thereafter, the fluid was redistributed over the cells every 15 minutes during the remaining incubation time.

Approximately 2.1×10^8 PFU was removed from the inoculum (Fig. 5). Since the culture contained approximately 10^8 cells, the ratio of bound virus to cells was 2.1 per cell. The total yield of virus in the fluid portion of the culture (slightly more than 10^{10} PFU) occurred at 9 hours. Since the virus was contained in 10 ml. of fluid,

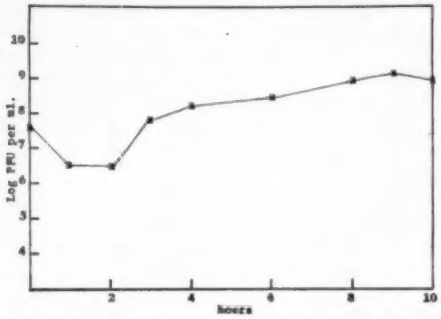

Fig. 5—Loss of infectious foot-and-mouth disease virus, type A, strain 119, from the fluid portion of a culture at 37 C. because of attachment of virus and thermal inactivation and the accumulation of virus in the fluid portion of each culture. Virus was liberated into 10 ml. of fluid which covered approximately 100 million cells.

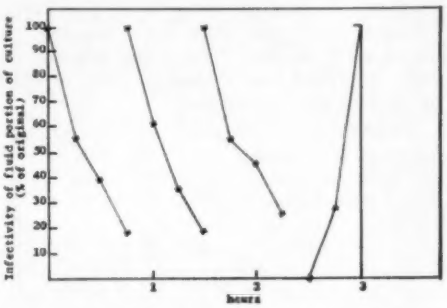

Fig. 6—Loss of infectious foot-and-mouth disease virus, type A, strain 119, from the fluid portion of a culture at 37 C. because of attachment of virus to the cells during a multiple inoculation. Three 5-ml. inoculums were applied to the cell sheet at intervals of 45 minutes. The culture contained approximately 80 million cells.

the concentration of the virus was slightly more than 10^9 PFU/ml.

Experiment 5.—The effect of multiple inoculation of bare cell layers was studied. Fluid was aspirated from Povitzky bottle cultures, and a 5-ml. inoculum of virus was added to each culture and distributed over the surface of the cells by repeated tiltings. Culture bottles were left upright for 30 seconds before each removal of fluid to allow fluid to drain from the cells. After 45 minutes' incubation, each virus inoculum was removed and replaced with another 5-ml. inoculum. At 1 hour and 30 minutes, this second inoculum was replaced with a third 5-ml. inoculum. The third inoculum was removed at 2 hours and 15 minutes, and the cell sheet in each bottle was rinsed twice with 100 ml. of Hanks' balanced salt solution warmed to 37 C. Ten milliliters of growth medium, containing 30% bovine serum warmed to 37 C., was added to each bottle; the cultures were incubated at 37 C. with the fluid redistributed over the cell surface every 15 minutes.

Thermal inactivation of the virus in each inoculum and the dilution of each inoculum by fluid that did not drain from the cells were determined. Each inoculum was diluted by the fluid that did not drain from the cells. As the result of this dilution, the concentration of the virus inoculums after addition to the cells was about 89% of that before addition to the cell sheet. Allowance was made for this in calculating the loss from the inoculums during incubation.

Results of this experiment (Fig. 6) are representative of the loss of virus from the inoculums because of attachment of virus to the cells. The amount of virus bound by the cells during each 45-minute incubation period was similar and amounted to approximately 80% of the virus that was added. Each 5-ml. inoculum contained 8×10^7 PFU. The 3 inoculums contained 2.4×10^8 PFU, of which about 1.9×10^8 PFU was bound by the cells. Since the culture contained about 80 million cells, the ratio of bound virus to cells was about 2.4 PFU per cell.

Virus accumulated rapidly in the fluid portion of the cultures after the 2-hour and

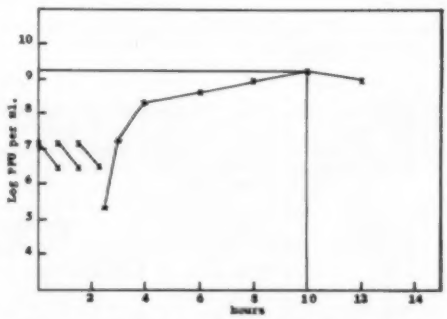

Fig. 7—Loss of infectious foot-and-mouth disease virus, type A, strain 119, from the fluid portion of a culture at 37 C. because of attachment of virus to the cells during a multiple inoculation and its accumulation in the fluid portion. Three 5-ml. inoculums were applied to the cell sheet at intervals of 45 minutes. Virus was liberated into 10 ml. of fluid which covered approximately 80 million cells.

15-minute washing (Fig. 7). Forty-five minutes after the cells were washed (3 hours after adding virus), the amount of virus liberated was at least as great as that in each of the inoculums.

Experiment 6.—A study of the accumulation of infectious virus in the cells and in the fluid portion of the culture was made. Fluid was aspirated from Povitzky bottle cultures. Five milliliters of virus (5×10^7 PFU) was added to each culture and distributed over the cell surface. After 45 minutes' incubation at 37 C., 5 ml. of growth medium containing 30% bovine serum was added. The inoculum was redistributed over the cell sheet every 5 minutes during the first 45 minutes of incubation. The fluid was redistributed over the cell sheet every 15 minutes during the remainder of the incubation period.

At 4 hours, and at every second hour thereafter throughout 24 hours' incubation, the cells and fluid from each of 2 cultures were harvested. The cells were scraped from the glass in each bottle with a rubber policeman equipped with a long-handled stainless-steel rod. The cells and fluid were transferred to a chilled conical tube and centrifuged at 2,000 r.p.m. (680 g) for 10 minutes in a holder containing chilled water. The fluid was aspirated from the cells, and the cells were rinsed in chilled Hanks' balanced salt solution. The cells were diluted 1:100 in chilled Hanks' solution and fragmented for 3 minutes in a chilled Omni Mixer or micro Waring Blendor bowl. Fluid was assayed for virus infectivity after centrifugation at 1,500 r.p.m. (380 g) for 15 minutes to remove cellular debris.

TABLE 2—Infectious Foot-and-Mouth Disease Virus, Type A, Strain 119, Recovered from the Fluid and Cells of 5-Liter Povitzky-Bottle Cultures of Bovine Kidney Cells after Addition of Virus to the Cells*

Hour post-inoculation	PFU fluid/total (%)	PFU cells/total (%)
4	55	45
6	57	43
8	73	27
10	86	14
12	90	10
14	90	10
16	90	10
18	90	10
20	90	10
22	90	10
24	90	10

* Each culture contained approximately 50 million cells covered by 10 ml. of fluid.

Comparative amounts of infectious virus in the fluid and cells were recorded (Table 2). At 4 hours, approximately 55% of infectious virus was recovered from the fluid. An increasing percentage of the virus appeared at longer incubation periods. At 12 hours, approximately 90% of the virus was recovered.

Titers of more than 10^9 PFU/ml. in the fluid portion and more than 10^{10} PFU/ml. in the cells were obtained from cultures containing 100 million cells.

Discussion

Infection of cells grown on glass by adding FMDV to the fluid portion of the culture has several disadvantages. The fluid portion not only dilutes the virus inoculum but acts also as a barrier to infection by suspending some virus away from the cells. Unless this virus is rapidly brought into contact with susceptible cells, much of it is lost through thermal inactivation. More rapid infection of large numbers of cells may be attained by increasing the virus concentration of the inoculum; however, this method for attaining virus attachment to a greater number of cells wastes the virus.

Virus in an inoculum added directly to the cell sheet is only slightly diluted and is brought nearer the cells. Working with dilute virus inoculums, Bachrach *et al.*[1] found that the plaque counts of FMDV were increased approximately sixfold when a given amount of virus was applied to the cell layers in a 0.1-ml. volume instead of a 6.4-ml. volume They attributed this, in part, to more effective collision between virus and cells when the more concentrated inoculum was used. The redistribution of a small volume of inoculum containing low concentrations of FMDV over the surface of the cells every 5 minutes during a 90-minute incubation period resulted in a twofold increase in the number of effective FMDV attachments to the cells. Adding a small volume of more highly concentrated virus directly to the cell sheet and redistributing it frequently was the most efficient method for rapidly infecting the cells. Approximately 80% of the virus in the inoculum was bound to the cells during a 45-minute incubation period when a 5-ml. inoculum was redistributed every 5 minutes over a sheet of cells covering approximately 75 square inches. Most rapid infection of cells

was obtained when multiple inoculums were employed.

Yields of high concentrations of virus in the fluid portion of the culture were obtained by reducing the volume of fluid in the culture while virus was being liberated from the cells. These concentrations were obtained when the volume of fluid covering a sheet of cells occupying 75 square inches was limited to 10 ml. Similar or slightly higher concentrations were obtained when this fluid was 5 ml. or 3 ml. However, if the cell sheet was allowed to drain before adding the fluid, this small amount of fluid was distributed over the cells with difficulty. For practical reasons, except during the period of attachment of virus, the volume of fluid above the cells was kept at 10 ml.

Concentrations of 10^{10} PFU/ml. were obtained within 6 hours after adding virus by fragmenting the cells when these methods for rapid infection of cells were employed. A concentration of $10^{9.2}$ PFU/ml. was obtained at 9 hours from the fluid portion of the culture. Peak concentrations might be reached earlier by increasing the infectivity of the inoculum beyond that employed in these studies. However, this might require special methods in preparing seed virus used in the inoculum.

Since thermal inactivation is an important factor in producing highly infectious FMDV-A119, the incubation time of this virus should be minimal. Methods for rapid infection of cells and the early harvest of the virus apply in producing virus of high infectivity.

The method of liberating virus by micro Waring Blendor or an Omni Mixer was superior to using various types of tissue grinders, to freezing and thawing, to treating with proteolytic enzymes, or to using combinations of these methods.

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SUMMARIO IN INTERLINGUA

Le Production de Alte Concentrationes de Virus de Febre Aphthose in Cytoculturas in Vitro

Esseva studiate le effecto de concentration, de inactivation thermic, de redistribution de inoculos, e del uso de multiple inoculos super le attachamento de virus de febre aphthose, typo A, racia 119 (VFA-A119) ad cellulas culturate super vitro. Le plus rapide infection de cellulas occurreva quando micre inoculos de alte infectivitate esseva addite directemente al superficie cellular e redistribuite omne 5 minutas durante le periodo de attachamento.

Le methodos usate pro le rapide production de alte concentrationes de VFA-A119 resultava in le production, in le portion cellular, de concentrationes de 10^{10} unitates a formation de placas per millilitro intra 6 horas e, in le portion liquide, de concentrationes de $10^{9.2}$ unitates a formation de placas per millilitro intra 9 horas post le addition de virus al cellulas.

Spontaneous Ileitis in Rats—A Report of 64 Cases

Robert G. Geil, D.V.M.; Charles L. Davis, D.V.M.; Samuel W. Thompson, D.V.M., M.S.

SUMMARY

A previously undescribed acute segmental ileitis was observed in 3 of 13 lots of albino rats purchased during a 5-month period. The condition, seen in 64 males of the Sprague-Dawley strain, was characterized by gross dilatation of segments of the ileum, in which hydropic degeneration of the smooth muscle with associated foci of coagulation necrosis occurred in the wall of the ileum. It appeared histologically dissimilar to ulcerative cecitis of rats, regional ileitis of man, and regional ileitis of swine.

INTRODUCTION

Regional ileitis is an established clinical and pathologic entity in man. The disease, first described in 1932,[2] is characterized by granulomatous inflammation of the submucosa of the ileum. Regional enterocolitis, histologically similar to regional ileitis of man, has been reported in Cocker Spaniels.[5] This disease process was localized primarily in the upper colon, although some lesions were also present in the ileum. A muscular hypertrophy of the ileum of swine also has been described.[3] It was believed to be either a primary idiopathic hypertrophy or secondary to dilatation due to a stenotic ileocecal orifice. A chronic cecal dilatation and ulceration, probably a manifestation of salmonellosis, has been reported in rats.[4]

Clinical and Histologic Observations

The condition herein reported affected 64 rats in 3 of 13 shipments of albino rats of the Sprague-Dawley strain, purchased during a 5-month period. Each shipment was composed of 170 to 180 males, 4 to 5 weeks old, within a weight range of 70 to 100 Gm. When the shipping crates were opened, several rats were seen to have severely distended abdomens (Fig. 1).

The affected rats had rough coats and some had diarrhea. Daily, during the 1st week to 10 days after arrival, several rats which had appeared normal upon receipt developed abdominal distension. The sick rats either died within 3 days after the development of abdominal enlargement or survived with the degree of abdominal distension slowly diminishing. In the 3 shipments, the morbidity varied from 2 to 17%, and the mortality was approximately 50% of the affected rats.

At necropsy, the most striking lesion seen was severe dilatation of the ileum, with 7.0- to 10.0-cm. segments distended to diameters up to 1.5 cm. (Fig. 2). In some cases, the aboral terminations of the dilatations were at the ileocecal orifice, while in others they were located as far as 4.0 cm. from this structure. The contents of the dilated ileum varied from a frothy semifluid to a pasty consistency. Other findings included yellow foci in the hearts or livers of about half the rats dying or killed within 4 days of the development of ileal distension. The mesenteric lymph nodes were usually enlarged and occasionally congested.

Specimens of heart, lungs, liver, spleen, stomach, normal and dilated ileum, cecum, colon, and kidneys were taken for histopathologic examination from rats dying naturally or killed when moribund. In addition, rats affected with the disease from 1 to 18 days were killed in order to study a progression of lesions. The tissues were fixed in 10% buffered neutral formalin. Paired specimens of liver and ileum were fixed in absolute alcohol. All tissue sections were stained with hematoxylin and eosin. Frozen sections of dilated ileum were stained with oil red O.

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All photographs and microphotographs are U.S. Army photographs taken by Mr. William Hommel of the Clinical Photography Laboratory, Fitzsimons General Hospital, Denver, Colo.

In 25 rats in which the condition had existed for less than 24 hours prior to death, the principal microscopic lesion seen in the gastrointestinal tract was focal or diffuse hydropic degeneration of the tunica muscularis of the dilated segment of ileum (Fig. 3). This degeneration was characterized by vacuolation of the smooth muscle cells and karyorrhexis. There was also increased eosinophilia of the cytoplasm of affected muscle fibers as compared to normal smooth muscle. In frozen sections, the vacuoles in the smooth muscle fibers did not stain with oil red O. There was also edema between the smooth muscle fibers. Extensive leukocytic inflammatory infiltrate, predominantly monocytic, was present in the muscularis. The myenteric plexuses of Auerbach and Meisner were masked by inflammatory cells in the dilated segment but were normal in the sections taken proximal and distal to the dilatation. The cellularity of the lamina propria was increased due to the presence of lymphocytic infiltrate. Many of the crypts of Lieberkuhn were occluded by amorphous masses of proteinaceous exudate (Fig. 4). The crypts were normal, deep to the point of plugging. Nondilated sections of ileum appeared normal.

In 25 rats killed 2 to 8 days after the onset of clinical signs, the degree of karyorrhexis and vacuolation of the muscularis was less than that seen in rats earlier in the course of the disease. Numerous smooth muscle fibers had undergone coagulation necrosis. In contrast to the monocytic infiltration seen in rats in the first 24 hours of the disease, a chronic inflammatory response, characterized by the presence of fibroblasts and early fibroplasia, was observed in the muscularis. The muscularis and lamina propria were also hyperemic. In the lamina propria, a lymphocytic infiltrate, similar to that described in rats earlier in the course of the disease, was also seen. However, proteinaceous material was not present in the crypts of Lieberkuhn. Moderate numbers of eosinophils were present in the muscularis of the rats surviving 4 days or longer after the onset of clinical illness. In several rats, the inflammation had extended to the serosa with the development of fibrous peritonitis. In one instance, a rat which had survived 4 days was found to have acute purulent peritonitis resulting from perforation of the wall of the ileum.

In addition to the ileitis, about half of the 30 rats dead during the first 4 days of illness had an acute focal myocarditis, characterized by hydropic degeneration of myocardial fibers and a moderate lymphocytic infiltration. Acute hepatitis with lymphocytic infiltration and focal coagulation necrosis of hepatic parenchymal tissue was also present in some of these rats. The areas of myocarditis and hepatitis corresponded to the yellow foci found in the hearts and livers during the gross necropsy. No cardiac or hepatic lesions could be found in tissue sections from rats dying or killed more than 4 days after the inception of the clinical disease. No microscopic lesion was seen in any section of spleen, kidneys, duodenum, jejunum, cecum, or colon obtained from affected rats at necropsy.

Although grossly dilated, the ileums of 14 rats killed after 8 or more days of clinical illness had only scant monocytic infiltrates in the otherwise normal-appearing smooth muscle layers (Fig. 5). Two of these rats had adhesions of the ileum to the liver and parietal peritoneum (Fig. 6). In the area of the adhesions, the overlying mucosa was ulcerated and the muscularis had been replaced by fibrous connective tissue. In 6 rats killed 14 days after the inception of clinical illness, only a slight dilatation of the ileum was observed. The muscularis in these rats appeared normal.

The disease never spread to rats of other lots, either males or females, which were kept in adjacent cages for 30 days. Six healthy 4- to 5-week-old males, placed in cages with an equal number of severely affected rats, were allowed to remain 20 days; none developed signs of clinical illness. Limited bacteriologic work was done in an attempt to rule out the possibility of salmonellosis. *Salmonella* could not be isolated, but cultures of paracolon bacteria and *Proteus* were obtained from the spleen, liver, and heart blood of 3 of 7 affected rats.

Discussion

From the series of lesions seen in sections taken from rats killed in different stages of the disease, we assume that this condition is primarily an acute, segmented hydropic degeneration of the smooth muscle of the wall of the ileum with associated foci of coagulation necrosis. Sections with severe hydropic degeneration of the tunica mus-

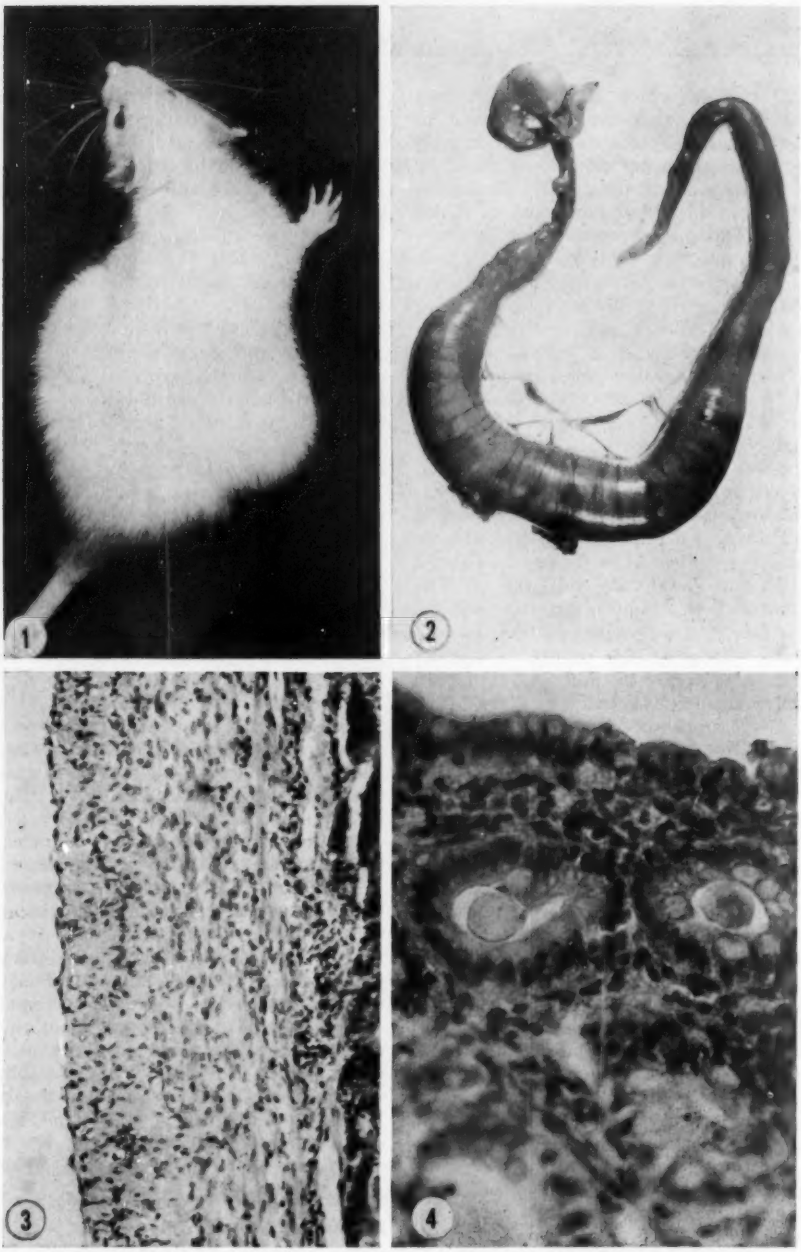

Fig. 1—Rat in which abdominal distension had developed 10 days prior to death. This rat was moribund when killed; at necropsy, a segment of ileum was found to be adhered to the jejunum. x ¾.

Fig. 2—Segment of the ileum from rat in which distension had developed 9 days prior to death. The 2 pieces of tissue adhered to the dilated segment of the ileum are portions of the ventral abdominal wall. x ½.

Fig. 3—Muscularis of ileum from a rat killed 12 hours after development of abdominal distension; karyorrhexis, hydropic degeneration, and monocytic infiltration. H & E stain; x 295.

Fig. 4—Ileum from a rat ill for 24 hours; occlusion of crypts of Lieberkuhn. H & E stain; x 700.

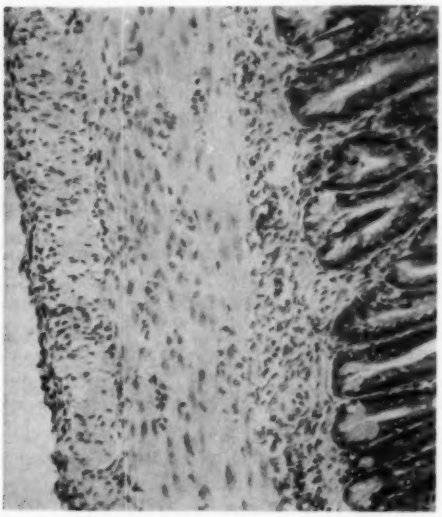

Fig. 5—Muscularis of ileum from a rat ill for 9 days; regeneration of smooth musc.e. H & E stain; x 295.

Fig. 6—Ulceration of ileum with adhesions to liver and diaphragm. This rat was ill for 10 days. H & E stain; x 6.

cularis and extensive monocytic infiltration, taken from rats with clinical cases of less than 24 hours duration, are believed to represent the acute stage of the disease. Examination of tissues from rats with cases of longer duration reveals what is believed to be progressive resolution, characterized by phagocytosis of cellular debris and regeneration of the smooth muscle fibers. The smooth muscle fibers of the muscularis are assumed to regenerate until, as seen in specimens taken 14 days or more after the inception of clinical illness, the gross and histologic appearance is normal except for areas underlying adhesions where fibrosis has occurred. The apparent origin of the condition in the muscularis and the absence of granulomatous inflammation would tend to differentiate it from both regional ileitis of man and the regional enterocolitis of dogs.[1,2,5]

Although dilatation was seen in ulcerative cecitis of rats, it was almost always limited to the cecum.[4] The disease also was more chronic and highly contagious. The inflammation originated in the mucosa and spread slowly through the muscularis to the serosa.

In the muscular hypertrophy of the ileum in swine, there is neither degeneration nor atrophy of the ileal smooth muscle.[3] It is interesting to speculate whether the disease described in swine could be a later stage of a condition similar to that described here, inasmuch as the cases in swine were seen after death from ileus or peritonitis. Possibly, because of the relatively small bowel diameter of the rat, the disease described here is of greater severity than that seen in swine and more frequently terminates in death. Such a postulation is based on the belief that the increased severity of regional ileitis in children can be attributed to a small bowel diameter.[5]

Histopathologic examination ruled out a similarity to Hirschprung's disease. Although Auerbach's and Meissner's plexuses are masked by inflammatory cells in the early stages of spontaneous regional ileitis in rats, they were normal distal to the dilatations and in the regenerated wall. Hirschprung's disease is characterized by degenerative changes in these plexuses.[1]

Early lesions in the muscularis, infrequency of ulceration, absence of mucosal erosion, and the inconstant diarrhea emphasize the dissimilarity of this condition to salmonellosis, human cholera, or typhoid.[1]

The frequent location of the aboral terminations of the ileal dilatations at distances from the ileocecal orifice indicates that ileocecal stenosis is not the causative factor in this condition.

The organisms isolated from the few rats submitted for bacterial culture are normal components of the intestinal flora. Their isolation raises the question of contamination or terminal bacteremia resulting from a devitalized intestinal wall.

The bacteriologic work done was not of sufficient scope to either establish or disprove the existence of an infectious etiologic agent for this condition. The significance of the observed occurrence of the disease only in males is unknown, since the number of male rats used by this laboratory exceeds females by a ratio of 10 to 1. Information as to the handling of the rats by the breeder or transporter is unavailable. Hence, what part, if any, the environment plays in this condition cannot be determined.

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SUMMARIO IN INTERLINGUA

Ileitis Spontanee in Rattos. Un Reporto de 64 Casos

Un previemente non describite acute ileitis segmental esseva observate in 3 de 13 lotes de rattos albin, comprate in le curso de un periodo de 5 menses. Le condition, vidite in 64 masculos del racia Sprague-Dawley, esseva characterisate per un grossier dilatation de segmentos del ileumn in que il occurreva degeneration hydropic de musculo lisie con associate focos de necrosis coagulational in le pariete del ileum. Histologicamente illo pareva dissimile a cecitis ulcerative de rattos, ileitis regional de humanos, e ileitis regional de porcos.

Characterization of 2 Viruses Obtained from Lymphomatous Liver

George R. Sharpless, Sc.D., and Erwin L. Jungherr, Vet. Dipl., D.M.V.

SUMMARY

Two viruses were obtained from a cell-free filtrate of lymphomatous liver from chicks infected with the RPL12 strain of "lymphomatosis" virus. One of the viruses was propagated by chick-to-chick passage of whole blood or serum from chicks intravenously inoculated with the filtrate. This virus produced typical erythroblastosis in chicks and in chicken embryos. Virus particles found in serum and in infected tissues had an approximate diameter of 100 mμ and a small nucleoid, 25 to 45 mμ in diameter. Particles found in tissues were either extracellular or, less frequently, intracytoplasmic.

The other virus was isolated in cell culture and attained a titer as high as 10^{10} per milliliter in cultures of various chicken embryo tissues. A crystal-like array of polygonal virus particles was found in the nucleus. Particles had a diameter of 86 mμ and a central core with a diameter of 44 mμ. In chicks, the virus was mildly pathogenic and produced areas of necrosis in the liver, often accompanied by cells with large basophilic intranuclear inclusion bodies. This virus was antigenically unrelated to that of lymphomatosis. Because of its similarity to the adenoviruses of man, the newly described virus was called Gallus adeno-like (GAL) virus. The findings suggest that some of the transmissible lymphomatosis virus preparations contained a contaminating virus (GAL) that is widespread, and whose presence might explain earlier reports of a primary necrotizing agent in transmissible avian lymphomatosis.

INTRODUCTION

Injection of a cell-free filtrate of lymphomatous liver into susceptible chicks induced visceral lymphomatosis manifested by either neural lymphomatosis or osteopetrosis.[3] To determine whether a single virus caused both manifestations, the filtrate was injected into chicken embryo liver cell cultures. The inoculated cultures yielded a virus not related to the agent of lymphomatosis. This new virus was named Gallus adeno-like (GAL) virus.[4,12] The same filtrate injected into chicks again caused lymphomatosis and, in the course of passage from chick to chick, erythroblastosis. The present report describes the morphology, site of propagation, and pathologic effect of both the GAL virus and the erythroblastosis agent.

Materials and Methods

The cell-free filtrate of lymphomatous liver from chicks infected with lymphomatosis strain RPL12 was furnished by Dr. B. R. Burmester, U.S. Regional Poultry Research Laboratory, East Lansing, Mich. White Plymouth Rock chicks, 3 weeks old, were inoculated intravenously with 0.2 ml. of a 1:10 dilution of 16th filtrate passage in chicks and were observed daily. Blood smears were made weekly.

Monolayers grown from trypsinized chicken embryo liver cells were inoculated with part of the filtrate and harvested when the cells were destroyed (3 to 5 days), and neutralization titers were calculated as previously described.[11] The GAL virus isolated from a plaque of the 15th serial cell culture passage of the original filtrate was used for pathologic studies. Two-week-old chicks, in groups of 5, were inoculated with 0.5 ml. of the isolate injected into the marrow space of the left femur. The 50% tissue culture infective dose ($TCID_{50}$) of each inoculum was $10^{-7.5}$ per milliliter. Chicks were killed for examination 6, 10, 13, or 17 days after inoculation.

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From Viral and Rickettsial Research, Lederle Laboratories, American Cyanamid Company, Pearl River, N.Y.

The authors thank William Boss, Ray Keenan, and Eugenia Berry for technical assistance; M.C. Davies and Mary Englert for the electron microscopy; Robert Wood for the photomicrographs; and Elisabeth Manoogian and Esther Chasan for help in preparing the manuscript.

Results

Chickens.—After 5 or 6 weeks, the smears from some chicks contained large lymphocytic cells and some erythroblasts. Heparinized blood from 1 of these chicks was injected intravenously into 15 3-week-old chicks. By comparing the time of the appearance of the first abnormal blood smears in the course of several passages, it was found that the incubation period decreased from 30 to 40 days to 10 to 15 days. Erythroblasts were most numerous in the blood from chicks with the shorter incubation period. In at least 30% of the chicks used for each passage, hemorrhagic foci, varying in diameter from less than 1 to 3 or 4 mm., were distributed throughout the skin, muscles, and viscera. The livers of these chicks were approximately normal in size, but the spleens were greatly enlarged. Some hemorrhagic areas, particularly in the viscera, resembled small tumors. Many of the chicks without extensive hemorrhagic foci or abnormal blood died later with greatly enlarged livers, and some with early blood changes appeared to recover but died in 2 or 3 months with visceral lymphomatosis.

The disease was routinely induced by intravenous injection of heparinized blood, but filtered plasma was also found to be infectious. With either blood or plasma, disease was induced less readily by the intramuscular or intracoelomic route than intravenously. Chickens 3 months old were as susceptible to infection as younger birds.

Chicken Embryos.—Embryos 10 to 12 days old were inoculated intravenously with 0.1 ml. of undiluted plasma infectious in chicks even in 1:1,000 dilution and having a high concentration of virus particles visible with the electron microscope. Most embryos died before hatching, many with gross lesions similar to those seen in chicks, *i.e.*, numerous hemorrhagic foci in the skin and muscle, enlarged spleens and numerous erythroblasts in the blood. Hemorrhagic foci appeared on the chorioallantoic membrane (CAM) and occasionally in the yolk sac. The lesions on the CAM frequently were cystic, with a gross appearance resembling caviar. Similar lesions were seen in some of the hatched chicks at 1 to 3 weeks of age. Intravenous injection of a 5% suspension of whole infected embryo, harvested when candled eggs disclosed dark lesions, was used for the passage from embryo to embryo. The virus could not be maintained by serial passage in the yolk sac or on the CAM, although such passage caused infection in some instances. By intravenous injection of 7-day embryos, the erythroblastosis agent was maintained for 13 serial passages.

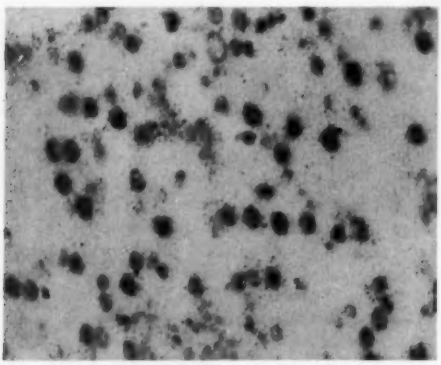

Fig. 1—Typical particles in sections of pellet from serum of chickens infected with erythroblastosis virus. x 31,667.

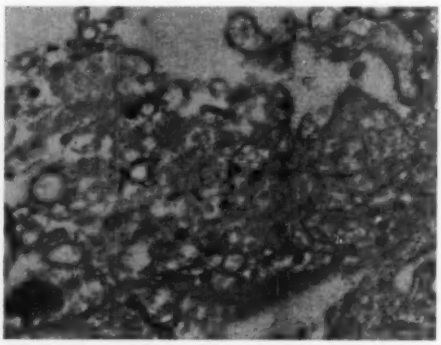

Fig. 2—Virus particles in spleen from chicken infected with erythroblastosis virus. x 15,067.

Tissue Culture.—A cytopathogenic agent was recovered from cell cultures inoculated with the original filtrate, and attained a titer of 10^9 to 10^{10} per milliliter in cultures of various cells of chicken embryos (whole embryo, liver, kidney, lung) but did not propagate in cells of other species. This agent was neutralized by antiserums proved earlier to neutralize lymphomatosis virus, and its 8th to 14th passages induced lymphomatosis. It therefore was thought to be the lymphomatosis virus.

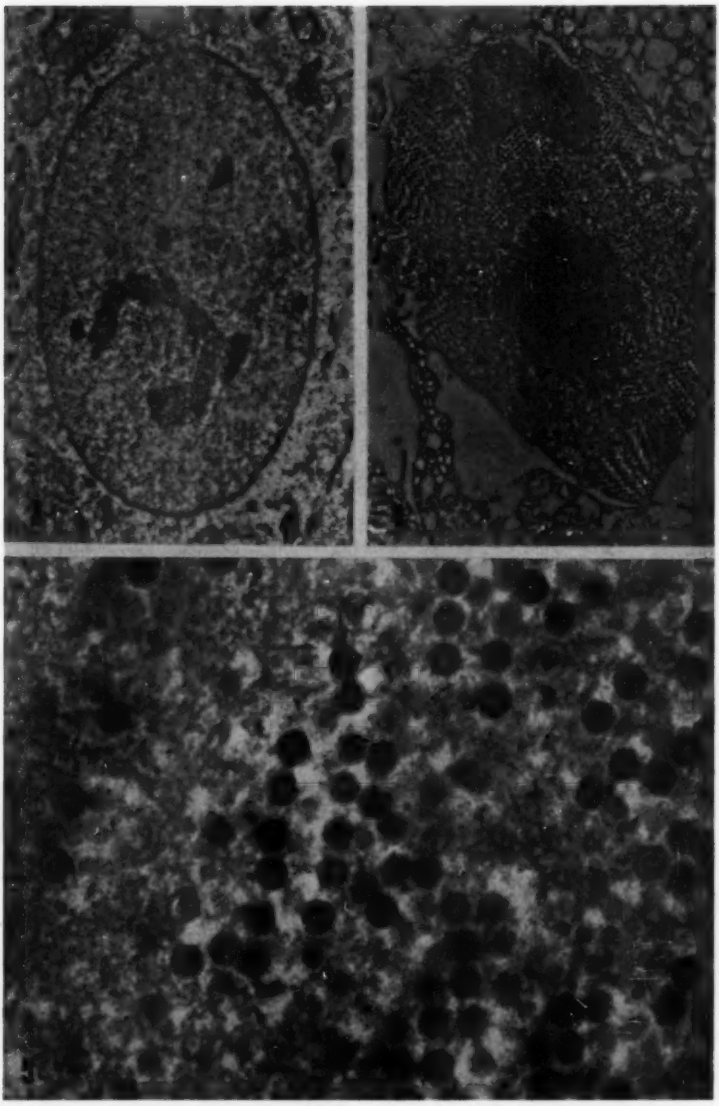

Fig. 3—Nucleus of chicken embryo liver cell culture 18 hours after infection with Gallus adeno-like virus; intranuclear formation typical of early stages of infection. x 10,000.

Fig. 4—Chicken embryo liver cell culture 42 hours after infection with Gallus adeno-like virus with necrotic cytoplasm and crystalline arrays of virus almost filling nucleus. x 10,000.

Fig. 5—Portion of Gallus adeno-like viral array in nucleus of chicken embryo liver cell 48 hours after infection; hexagonal cross-section of viruses and internal structure. x 72,200.

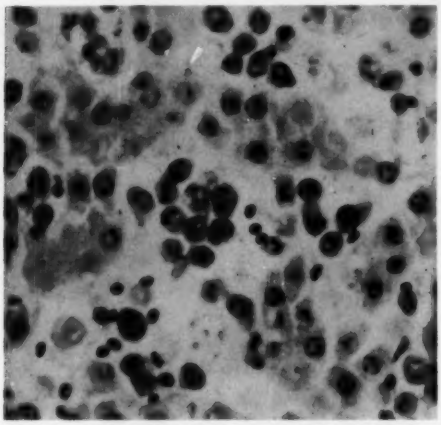

Fig. 6—Liver of chick inoculated with erythroblastosis agent; paraffin section with typical early leukostasis. H & E stain; x 333.

However, the virus was not always recoverable from lymphomatosis tissues and, whether isolated from plaques, from cell cultures, or grown from earlier passages after prolonged storage, it failed to produce lymphomatosis. Chickens vaccinated with formalin-inactivated suspensions of infected chicken embryo or tissue culture fluid harvest remained susceptible to lymphomatosis, and the progeny of dams inoculated with the virus were as susceptible to lymphomatosis as the progeny of untreated dams. While these results indicated that this particular virus was not the etiologic agent of lymphomatosis, the characteristics of the virus were of interest because it appeared to be widespread in chickens and could induce pathologic changes in hatched chicks.

Cell cultures infected with the new virus produced some cytopathic effects in 24 hours and reached maximum titer in 48 to 72 hours. The virus was formed in the cell nucleus 12 to 18 hours before it was released. It was called GAL virus because of its resemblance to the adenoviruses of man in wide distribution, morphology, resistance to ether,[12] and propagation within the nucleus.

Chicks inoculated in the femur with GAL virus had only mild signs of disease. Some drowsiness occurred on the 10th to 12th day after inoculation, and combs and wattles lost color. These signs lasted for 3 to 5 days, and by the 17th day all chicks appeared healthy. In this experiment no chicks died, but in other experiments as many as 25% died, with no gross abnormalities other than anemia.

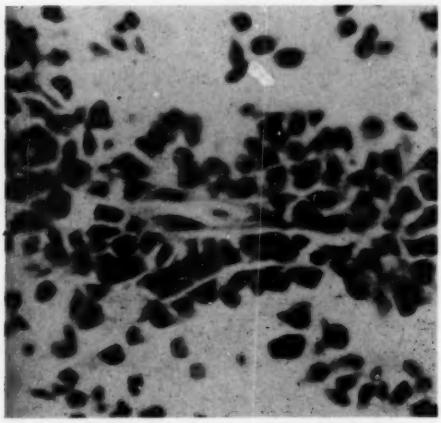

Fig. 7—Bone marrow of chick inoculated with erythroblastosis agent; paraffin section with neoplastic transformation of endothelial cells into erythrogonia, also extravascular myelocytes and general hyperplasia. H & E stain; x 333.

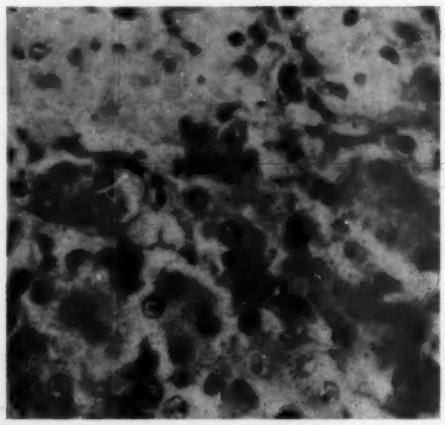

Fig. 8—Liver of chick inoculated with Gallus adeno-like agent; paraffin section with area of necrosis (upper left quadrant). H & E stain; x 333.

Electron Microscopy.—Erythroblastosis.—When pellets obtained by high-speed centrifugation of infected plasma were sectioned and examined, many virus-like particles were seen (Fig. 1). Similar particles

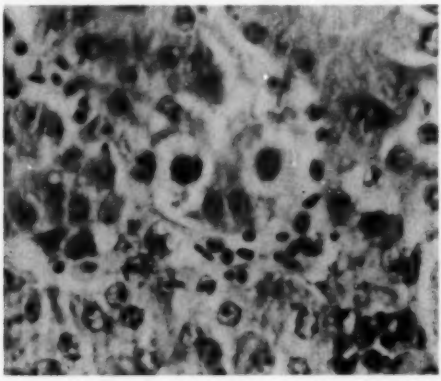

Fig. 9—Liver of chick inoculated with Gallus adeno-like agent; paraffin section with basophilic intranuclear inclusion bodies in 2 cord cells (center). H & E stain; x 333.

were seen in sections of skin lesions from infected chicks and in visible lesions and enlarged spleens of infected embryos (Fig. 2). Particles were most abundant in intercellular spaces, often where cells appeared to have disintegrated; they also were seen in the cytoplasm, often apparently in the mitochondria, but were not seen in the nucleus. The particles averaged about 100 mμ in diameter and had an electron-dense central core 25 to 45 mμ in diameter. In size and in location within the cytoplasm, the particles seen in plasma and tissue sections conform to the descriptions of erythroblastosis virus by Bonar *et al.*[2] and Dmochowski *et al.*[8]

Gallus Adeno-like Virus.—In the nucleus of infected cells an accumulation of electron-dense material was seen by 18 hours (Fig. 3), and there were some virus particles in various parts of the nucleus by 24 hours. By 48 hours (Fig. 4), most of the nucleus was packed with virus particles in a crystal-like arrangement. At this stage, the cytoplasm was disintegrating, but no virus particles were seen there. In cross section, the virus was hexagonal (Fig. 5). Subsequent work indicated that the virus was an icosahedron,[5] had a double membrane and a nucleoid, and averaged 86 mμ in diameter with a central core 44 mμ in diameter.[6, 12]

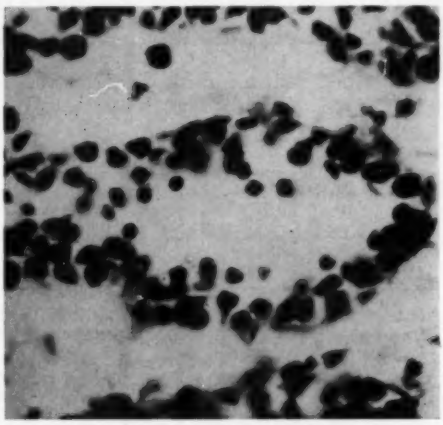

Fig. 11—Bone marrow of chick inoculated with Gallus adeno-like agent; paraffin section with hypertrophy and hyperplasia of endothelial cells but overall hypoplasia. H & E stain; x 333

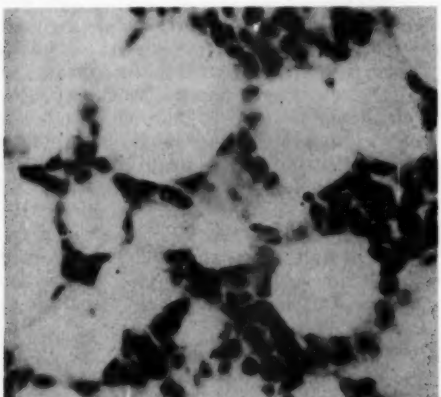

Fig. 10—Bone marrow of chick inoculated with cell culture fluid; paraffin section with normal intravascular erythropoiesis, extravascular fat cells, and heterophils. H & E stain; x 333.

Pathology.—In general, the disease caused by the erythroblastosis agent was grossly characterized by enlargement of the spleen and a "currant jelly" appearance of the bone marrow,[9] while the GAL virus often failed to induce definite alterations of the spleen or bone marrow, or any gross changes other than occasional yellowish discoloration of the liver.

Erythroblastosis.—Characteristic microscopic changes consisted of leukostasis in the sinusoids of the liver (Fig. 6). Cellular elements were represented almost exclusively by immature erythroblasts, so-

called erythrogonia. Leukostasis varied in degree in different areas, from minor to microtumor-like aggregates. Liver cord cells were normal or had slight evidence of compression damage. In the spleen, the adenoid sheaths were essentially normal, even in advanced stages of the disease, but the red pulp was thickened as a result of leukostasis in the capillaries. This change probably accounted for the grossly recognizable splenomegalia. The bone marrow was best studied in the early stages of the disease, at the time of initial neoplastic transformation of endothelial cells into erythrogonia (Fig. 7).[10] As this process advanced, the entire capillary wall became lined with erythrogonia which eventually overflowed the vascular confines, compressing the extravascular fat spaces and causing an intense hyperplasia.

Gallus Adeno-like Infection.—The early stage was characterized by isolated areas of eosinophilic necrosis in the liver, with lysis of the cytoplasm of affected cells and no major damage to the nuclei (Fig. 8). This was followed by definite pyknosis or karyolysis. Necrotic foci were frequently band-shaped and had no characteristic relationship to the architecture of the hepatic lobule. Initial necrotic foci were often seamed by cells, either hepatic cord or Kupffer cells, which stood out because of their large basophilic or amphophilic intranuclear inclusion bodies (Fig. 9), resembling those found by Defendi and Sharpless [7] in chicken embryos inoculated with the RPL12 "lymphomatosis" virus. In favorable examples, the chromatin on the nuclear membrane was marginated.

The spleen was grossly normal in size and appearance but had microscopic evidence of early necrosis of the adenoid sheath cells. This change was characterized by either vacuolar degeneration of these cells or, in some instances, by extrusion of a coagulum which gave the cells a signet-ringlike structure. In general, the adenoid sheaths were enlarged, but the red pulp was compressed and apparently diminished in volume, giving a relative proportion of white to red pulp inverse to that in erythroblastosis.

Here, too, the bone marrow was best studied in the early stages. The endothelial cells of the sinusoids proliferated without evidence of neoplastic transformation.

TABLE 1—Comparative Histologic Features of Erythroblastosis and Gallus Adeno-like Infection

Location	Erythroblastosis	Gallus adeno-like infection
Liver	Leukostasis	Focal necrosis, basophilic intranuclear inclusion bodies.
Spleen	Normal adenoid sheaths, thickening and leukostasis in red pulp.	Early necrosis of adenoid sheath cells, thinning of red pulp.
Bone marrow	Neoplastic proliferation of endothelial cells, extravascular overflow, hyperplasia.	Non-neoplastic proliferation of endothelial cells, extravascular hypoplasia.

While the proliferated cells conformed in all respects to normal endothelial cells (Fig. 10), they sometimes were seen in multiple layers (Fig. 11). The extravascular space, which normally in young chickens is rich in heterophils, was hypoplastic and contained only scattered remnants of reticulum cells and other agranular monocytic elements.

Discussion

The differential histopathologic features of erythroblastosis and GAL infections are clear when presumably pure agents are considered, but under field conditions histopathologic expressions of both agents are apt to be observed. Asplin [1] worked with a "lymphomatosis agent" which produced focal necrosis in the liver in the early stages and, later, proliferative neoplastic changes. Although the agents cannot be compared today, earlier observations of agents with the pathologic expressions of GAL virus are suggested by the literature. The apparently widespread occurrence of GAL may require a revision of earlier pathologic concepts.

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SUMMARIO IN INTERLINGUA

Characterisation de 2 Virus Obtenite ab Hepate Lymphomatose

Duo virus esseva obtenite ab un filtrato acellular del hepates lymphomatose de gallinettas inficite con le racia RPL12 de virus de "lymphomatosis." Un del virus esseva propagate per le passage ab gallinetta ad gallinetta de sanguine total o sero de gallinettas inoculate intravenosemente con le filtrato. Iste virus produceva typic erythroblastosis in gallinettas e embryones de gallina. Particulas del virus trovate in sero e in inficite tissus habeva un diametro de approximativemente 100 mμ e un micre nucleoide de 25 a 45 mμ. Le particulas trovate in tissus esseva extracellular o, minus frequentemente, intracytoplasmic.

Le altere virus esseva isolate in cytocultura e attingeva un titro de usque a 10^{10} per millilitro in culturas de varie tissus de embryon de gallina. Un aggruppation crystalloide de particulas polygone de virus esseva trovate in le nucleo. Iste particulas habeva un diametro de 86 mμ e un massa central con un diametro de 44 mμ. In gallinettas iste virus esseva levemente pathogenic e produceva areas de necrosis in le hepate, frequentemente accompaniate de grande basophilic corpores de inclusion intranucleari. In tests de antigenicitate, iste virus se monstrava non-relationate con illo de lymphomatosis. A causa de su similitude con le adenoviruses del homine, le novemente describite virus esseva appellate virus Gallus adeno-like (GAL). Le constatationes suggere que certes del preparatos de transmissibile virus de lymphomatosis contine un virus contaminante—i.e. virus GAL—que es extensemente distribuite e le presentia del qual explica possibilemente previe reportos de un agente primari de necrotisation in transmissibile lymphomatosis avian.

Isolation of Clostridia by Crystal Violet Inhibition of Aerobic Spore-Forming Bacteria

Donald C. Blenden, M.A., D.V.M., and Charles P. Merilan, M.A., Ph.D.

SUMMARY AND CONCLUSIONS

The inhibitory effect of crystal violet, both in agar and thioglycollate broth, was determined for 5 strains of *Bacillus*. Only one of these strains was found to have marked resistance to the dye. Crystal violet tolerance of the *Bacillus* was related to the age of the culture used, probably because of spore formation. Thirteen species of *Clostridium* were found to be more resistant to crystal violet than were bacilli. The only exception was the case of aged *Bacillus* cultures which had an extremely high tolerance to crystal violet. Two procedures for the isolation of *Clostridium* by selective inhibition of contaminant organisms were used. Under the condition of these experiments, the dye procedure greatly facilitated the isolation of clostridia from both tissue and fecal specimens.

INTRODUCTION

The need for a practical and dependable technique for the isolation of clostridia has never been completely satisfied. Such a technique should be easily adapted to a large number of *Clostridium* species and should require no special procedures for its use as a routine laboratory method. Many contaminating organisms, especially the facultative anaerobic bacilli with their spreading growth on agar plates, have hindered the development of a satisfactory isolation procedure for members of the genus *Clostridium*. Members of the genus *Bacillus*, by virtue of cellular morphology, staining reaction, *beta* hemolysis of blood, colonial appearance, and facultative growth, closely resemble the clostridia. Thus, selective inhibition of the bacilli would facilitate the isolation of clostridia from clinical specimens as well as from soil and other sources. This investigation was undertaken to develop a practical technique for the isolation of clostridia in the presence of contaminating bacilli.

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From the Departments of Veterinary Bacteriology and Parasitology and Dairy Husbandry, University of Missouri, Columbia. Contribution from the Missouri Agricultural Experiment Station series No. 2232, approved by the director.

Review of Literature

Many reports concerned with the isolation and identification of clostridia have appeared in the literature. One technique utilized a precipitin method for rapid identification of clostridia as a major factor in the process of isolation.[10] The success of this procedure, involving the incorporation of a specific antiserum in the agar and selection of colonies with zones of precipitation, is sometimes hampered by its inherent specificity and overgrowth of contaminating organisms. Similar difficulties may arise in using the method in which various species of clostridia are characterized by their lecithinase activity which causes zones of precipitation in egg yolk agar.[8]

A more general procedure was presented in which Wilson-Blair medium helped bring about primary isolation of clostridia.[7] In this medium, however, the colonies of some *Clostridium* species are difficult to distinguish from those of contaminant organisms. Also, a medium containing acetic acid has been used for the isolation of clostridia from heavily contaminated specimens.[9] This medium was found to selectively inhibit some members of the enteric group of organisms.

Another procedure for isolating clostridia involved the inoculation of a variety of mediums from each specimen,[11] but even under these conditions, the investigators found that contaminating organisms presented a considerable problem. Several workers [2, 5] have used heat treatment to isolate clostridia from specimens containing bacterial contaminants. This technique, although effectively eliminating nonsporulating organisms, has the obvious disadvantage of destroying all clostridia which have not sporulated and, in addition, not destroying the spores of *Bacillus* contaminants.

Members of the genus *Bacillus* have long been a general source of difficulty in the isolation of otherwise pure cultures, even though as early as 1912 it was indicated that gentian violet was bacteriostatic for bacilli.[1] This work was substantiated in 1918 by utilizing gentian violet in a concentration of 1:100,000 to selectively inhibit the bacilli.[4] In 1942, it was reported that all aerobic gram-positive organisms had low resistance to crystal violet.[3] Also, in 1951, it was found to be

superior to other dyes for inhibiting bacilli in the isolation of *Mycobacterium tuberculosis*.[6]

Materials and Methods

Preliminary studies substantiated the fact that crystal violet was more toxic for members of the genus *Bacillus* than for *Clostridium* spp. In order to more accurately evaluate these observations, 9 different strains of *Bacillus* were tested for their ability to grow anaerobically. The 5 strains which gave good growth under these conditions were then compared for their tolerance to crystal violet. One strain of *Bacillus subtilis* was found to have much greater resistance to the inhibitory effect of the dye and, therefore, was selected as the *Bacillus* test strain. Thirteen species of *Clostridium*, representing some of the most commonly found saprophytes and pathogens, were utilized for the investigation. Mixed cultures were prepared by adding 1 ml. of a 48-hour *Clostridium* spp. culture to 10 ml. of a tissue or fecal specimen or to a 48-hour *B. subtilis* culture.

Two basic mediums were used in this study. They were bacto-fluid thioglycollate medium and tryptose agar.* Varying amounts of a 1:1,000 dilution of crystal violet stock solution were added to these mediums to obtain the desired concentrations to be tested. The crystal violet used had a total dye content of 97%. Following the preparation of the mediums and the addition of the crystal violet, the mediums were sterilized in the usual manner. Broth cultures were inoculated by loop or by pipette. These were then incubated at 37 C. Crystal violet agar plates were inoculated by the usual streaking technique and were placed in a Brewer anaerobic jar. The jar was evacuated, flushed 3 times with natural gas, and catalyzed for 30 minutes by the platinum catalyzer in the jar lid. The jar was then placed in an incubator at 37 C. for 48 hours.

Observations and Results

The inhibitory effects of various concentrations of crystal violet in agar for *B. subtilis* and 13 species of *Clostridium* were determined. It was found that the addition of crystal violet to agar in a concentration of 1:750,000 would selectively inhibit growth of the *Bacillus* without markedly affecting the clostridia. This strain of *B. subtilis* was found to give growth at a crystal violet concentration of 1:1,750,000 or less.

A separate experiment was conducted in which mixed cultures of clostridia and *B. subtilis* were inoculated on agar plates having a crystal violet concentration of 1:750,000. Plain agar plates were inoculated as controls. Isolated colonies of clostridia were consistently observed. Even in those instances where *B. subtilis* was not completely inhibited, its growth was greatly restricted, and no difficulty was met in making pure culture isolates of *Clostridium*. On control plates there was uniform spreading growth of *B. subtilis*, so that isolation of the clostridia was impossible.

The possibility of using crystal violet thioglycollate broth as a selective enrichment broth to obtain increased *Clostridium* cell counts before isolation was also investigated. The *Clostridium* spp. were more resistant to the effects of the dye in thioglycollate broth than in agar. The crystal violet tolerance of *B. subtilis* in broth was found to be associated with the age of the culture when inoculated. Redetermination of the critical concentrations of crystal violet in agar for *B. subtilis* indicated that only advanced culture age markedly influenced the crystal violet tolerance of this organism when grown on crystal violet agar plates. It was found that when 24- or 48-hour cultures were used for inoculum, the *B. subtilis* would grow only in crystal violet dilutions of 1:1,750,000 or greater. However, when a 7-day culture was used as an inoculum, the *B. subtilis* would grow in a crystal violet concentration of 1:250,000. Thus, for comparative purposes, 48-hour *Bacillus* and *Clostridium* cultures were used in preparing a mixed culture to be grown in crystal violet thioglycollate broth. As indicated (Table 1), the use of crystal violet thioglycollate broth as a primary selective enrichment medium materially aided in the isolation of clostridia from crystal violet agar plates. *Bacillus subtilis* colonies were usually not present, but those that grew were sharply inhibited in numbers and size. Tryptose agar plates which did not contain crystal violet were inoculated with the same culture mixtures as controls. In each case, the spreading growth of the *B. subtilis* resulted in the contamination of all *Clostridium* colonies so that single colony isolates could not be made.

To test the practical application of these methods, a preliminary study was made by inoculating bovine feces with *Clostridium novyi* and *Clostridium sporogenes*. These clostridia were easily recovered on primary isolations on crystal violet agar plates with no evidence of contaminants with spreading growth. Also, samples of tissue suspected of being infected with clostridia were inoculated into crystal violet thioglycollate

* Difco Laboratories, Inc., Detroit, Mich.

TABLE 1—Growth of Clostridia-Bacillus Mixed Cultures on Crystal Violet Agar After Incubation in Crystal Violet Thioglycollate Broth

Organism (plus *B. subtilis*)	Crystal violet plates		Control plates (no crystal violet)
	1:750,000	Remarks	
Cl. bifermentans	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. botulinum	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. sporogenes	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. histolyticum	++	Isolated *Clostridium* colonies*	*B. subtilis* covered plates
Cl. tetani	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. novyi	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. tetanomorphum	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. sphenoides	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. septicum	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. perfringens	+	Isolated *Clostridium* colonies*	*B. subtilis* covered plates
Cl. difficile	++	Isolated *Clostridium* colonies	*B. subtilis* covered plates
Cl. hemolyticum	+	Isolated *Clostridium* colonies*	*B. subtilis* covered plates
Cl. chauvei	+	Isolated *Clostridium* colonies*	*B. subtilis* covered plates

Plate inoculum was taken from crystal violet thioglycollate broth tube of the maximum concentration showing growth. ++ = Uninhibited growth of *Clostridium*; + = partly inhibited growth of *Clostridium*.
* *Bacillus* colonies present, but sharply inhibited.

broth and on crystal violet agar plates. *Clostridium chauvei* and *Clostridium septicum* were isolated from these clinical specimens on several occasions.

Discussion

The isolation of clostridia in pure culture is difficult in many cases, because contaminating organisms are culturally similar to the clostridia. The results of this investigation indicated that an isolation procedure involving the use of crystal violet materially reduced and in many instances eliminated difficulties arising from the presence of facultative *Bacillus* spp.

Only 1 of 5 laboratory *Bacillus* strains was found to have an appreciable tolerance for crystal violet, and with but 1 exception this tolerance was significantly lower than that of 13 species of *Clostridium*. The exception, that the *Bacillus* became more resistant to crystal violet, was presumably due to sporulation of the *Bacillus* culture prior to testing, because stained smears of the aged cultures revealed the presence of large numbers of spores. Also, 24- to 48-hour cultures did not develop this increased crystal violet tolerance, thus largely eliminating the possibility of adaptive enzyme formation as the mechanism of increased resistance. Metabolic activities of the newly developed vegetative cells seemed to inactivate a portion of the crystal violet and thus gradually reduced the effective concentration of the dye to the point that vegetative cells developed from other spores were not inhibited.

On the basis of the results of this investigation, the following procedures were found to have application for the isolation of clostridia: (1) Using a loop, streak the inoculum directly from the specimen onto agar containing crystal violet in a concentration of 1:750,000. Incubate these plates anaerobically for 48 hours prior to subculturing typical *Clostridium* colonies. (2) Where the specimen is suspected of containing relatively few clostridia, place a heavy inoculum in freshly heated and cooled thioglycollate broth tubes containing crystal violet in a concentration of 1:250,000. The inoculum should be introduced into the lower portion of the tubes with a minimum of agitation to maintain anaerobiosis. Incubate broth cultures for 48 hours, and then streak the inoculum from these tubes onto crystal violet agar plates for colonial isolation as described.

Although these procedures have not brought about complete elimination of all contaminating organisms, the results of their use on clinical or other heavily contaminated specimens indicate that they greatly facilitate the isolation of clostridia. The colonies other than clostridia are sharply reduced in numbers, and the spreading type of growth of many organisms is markedly suppressed or completely inhibited. The possibility exists that the use of a medium containing crystal violet to inhibit the aerobic spore formers and acetic acid to aid in the inhibition of the enteric organisms would be of great benefit. The reliability of this technique must be studied further, using comparative methods on clinical material. Also yet to be determined is whether there are strain differences in the susceptibility to crystal violet in the species of *Clostridium* as there are in the bacilli.

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SUMMARIO IN INTERLINGUA

Isolation de Clostridios per le Inhibition, per Medio de Violetto Crystallisate, de Aerobie Bacterios Sporophore

Le effecto inhibitori de violetto crystallisate, tanto in agar como etiam in bouillon a thioglycollato, esseva determinate pro 5 racias de *Bacillus*. Esseva trovate que solmente un de iste racias habeva un resistentia marcate contra le colorante. Le grado de tolerantia pro violetto crystallisate esseva relationate al etate del cultura de *Bacillus* usate, probabilemente a causa del formation de sporas. Pro 13 species de *Clostridium* un resistentia contra violetto crystallisate plus grande que illo del bacillos esseva demonstrate. Le sol exception esseva culturas de *Bacillus* de alte etates que habeva un extreme grado de tolerantia pro violetto crystallisate. Essva usate duo technicas pro le isolation de *Clostridium* per inhibition selective de contaminante organismos. Sub le condition de iste experimentos, le technica a colorante facilitava grandemente le isolation de clostridios tanto ab tissu como etiam ab specimens fecal.

Scientific Notes

Establishment of Permanent Carotid Loops in Goats

S. K. Jha, G.B.V.C., M.S.; W. V. Lumb, D.V.M., Ph.D.; R. F. Johnston, D.V.M., Ph.D.

SUMMARY

Carotid loops established in 10 goats remained patent for a minimal period of 3 months. In 1 it was still functioning after 9 months. After the surgical sites had healed, 3 goats died from hemorrhage following loop injury.

INTRODUCTION

Arterial blood is frequently required for the determination of the arterial level of metabolites. Graham [1] described a method of carotid loop formation for obtaining arterial blood from the goat. McClymont [2] described a method for establishing carotid loops in cattle. The authors established permanent carotid loops in goats for measuring arterial blood pressures and recording pulse rates.

Materials and Methods

Ten goats of various ages, all of which were mature females of mixed breeding, were used. Their body weights varied from 40 to 110 lb. Some were pregnant. Feed was withheld from each goat for 24 hours and, about 4 hours before surgery, 1 oz. of an antifermentative drug* was administered through a stomach tube to retard rumen fermentation. Ten minutes before operation, the goat was given a tranquilizer** at the rate of 1 mg. per lb. of body weight, intravenously in the cephalic or the saphenous vein.

The left side of the neck was then clipped, and the goat was secured to the operating table in right lateral recumbency. Atropine sulfate (1/75 gr. per 10 cc. solution) was mixed with a 4% thiamylal sodium.† This mixture was injected intravenously for effective anesthesia and prevention of excessive salivation. After the goat was anesthetized, the site of operation was thoroughly scrubbed with liquid germicidal detergent.‡ Aseptic technique was followed throughout the operative procedure.

The external jugular vein was raised by digital pressure, and a skin incision approximately 4 inches long was made immediately dorsal to its upper limit. This incision was at the junction of the middle and lower thirds of the jugular furrow. By blunt dissection, the sternocephalicus was separated from the omohyoideus, and the carotid artery was exposed. The artery was separated from the structures of the carotid sheath, and any smaller vessels were ligated and cut close to the main vessel. Physiologic saline solution was used on the artery to prevent drying. A 2nd incision was made ventral and parallel to the 1st, and the consequent strip of skin was then wrapped around the artery to form a sheath, open along the medial side. The 2 free edges of the sheath were then sutured together, after which the 2 free edges of the skin, left after formation of the sheath, were sutured. Care was taken to obtain good approximation at the junction of the loop with the adjoining skin surface.

Fig. 1—Loop, after 9 months.

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From the Department of Surgery and Medicine, Michigan State University, East Lansing.

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* Turcapsol, Pitman-Moore Co., Division of Allied Laboratories, Inc., Indianapolis, Ind.

** Vetame (triflupromazine hydrochloride), E. R. Squibb and Sons, New Brunswick, N.J.

† Surital, Parke, Davis & Co., Detroit, Mich.

‡ Active ingredient benzethonium chloride, Parke, Davis & Co., Detroit, Mich.

Results and Discussion

Results of loop formation were very satisfactory. The loop remained patent in all goats for a minimal period of 3 months. In 1 goat, it was still functioning after 9 months (Fig. 1). After the surgical sites had healed, 3 goats died from loop hemorrhages. In 1 goat, a piece of wire attached to the goat's collar was thought to have caused perforation of the carotid artery. A horned goat was found to butt the others, and this may have caused damage to the loops, resulting in hemorrhage in the other 2 goats.

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SUMMARIO IN INTERLINGUA

Establimento de Permanente Ansas Carotic in Capras

Ansas carotic establite in 10 capras remaneva patente durante periodos de al minus 3 menses. In 1, le ansa functionava ancora post 9 menses. Post que le sitos chirurgic esseva resanate, 3 capras moriva ab hemorrhagia causate per injurias del ansas.

Natural Occurrence of Leptospirosis in the Meadow Vole, Microtus pennsylvanicus

Lawrence G. Clark, D.V.M.; Joseph I. Kresse, B.S.;
Robert R. Marshak, D.V.M.; Charles J. Hollister, D.V.M.

During the course of epizootiologic studies of leptospirosis in southeastern Pennsylvania, Leptospira ballum was isolated from a meadow vole, Microtus pennsylvanicus. Although Microtus species are important leptospiral reservoirs in Russia,[2, 3] Israel,[6] Europe,[7] and the Far East,[7] this is the first report of a leptospiral isolation from Microtus in the United States.

Eight specimens of Microtus pennsylvanicus were obtained in traps* from the premises of a dairy farm on which cattle were known to be infected with leptospirosis. The serotype infecting the herd has not been definitively identified, but is known to be a member of the L. hebdomadis serogroup.

The trapped voles were transported alive to the Leptospirosis Laboratory, University of Pennsylvania, where they were killed in a chloroform chamber, dipped in aqueous disinfectant,** pinned to a board, and sprayed with an alcoholic solution of the disinfectant. Kidneys were removed aseptically and ground in TenBroeck tissue grinders. Stuart's liquid medium base† was added to approximate a 10% suspension.

One to 3 drops of the kidney suspension was inoculated into 1 tube of each of the following mediums: Fletcher's semisolid medium (Difco) with 10% rabbit serum, Fletcher's semisolid medium with 15% horse serum, Chang's semisolid medium[1] (Hamilton, Montana, modification[4]) with 10% rabbit serum, Chang's semisolid medium with 15% horse serum, and Stuart's semisolid medium[5] with 10% rabbit serum.

Serial tenfold dilutions of the kidney suspension were made with Stuart's liquid medium base to approximate 10^{-1} through 10^{-5} final dilutions. One to 3 drops of each dilution was inoculated into a tube of Fletcher's semisolid medium with 10% rabbit serum.

All culture tubes were incubated at 29 C. and examined at 10- to 14-day intervals by darkfield microscopy. Growth was detected on postinoculation day 39 in tubes inoculated with kidney material from vole 386, a mature male. On this date, leptospires were observed in Chang's medium with 15% horse serum, Chang's medium with 10% rabbit serum, and 10^{-1} through 10^{-3} dilutions of Fletcher's medium with 10% rabbit serum. Subcultures from tubes of Fletcher's semisolid medium with 10% rabbit serum were sent to the Leptospirosis Unit, Animal Disease Eradication Diagnostic Laboratory, Ames, Iowa, where they were identified as Leptospira ballum.

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From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia. Dr. Clark and Mr. Kresse are assigned to the School of Veterinary Medicine, University of Pennsylvania, by Laboratory Services, Animal Disease Eradication Division, ARS, USDA.

* Sherman Live Traps, H. J. Spencer & Sons, Gainesville, Fla.

** Benzalkonium chloride technical solution, 10% (Winthrop Laboratories).

† Difco Laboratories, Detroit, Mich.

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SUMMARIO IN INTERLINGUA

Occurrentia Natural de Leptospirosis in le Campaniol Commun (*Microtus pennsylvanicus*)

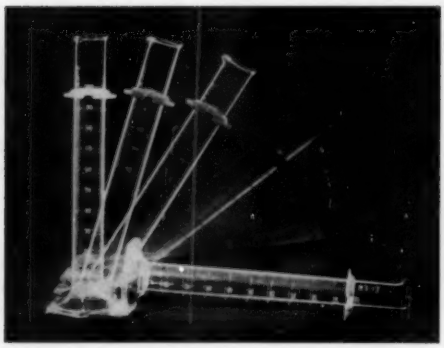
In le curso de studios epizootiologic de leptospirosis in Sud-Est-Pennsylvania, Leptospira ballum esseva isolate ab un campaniol commun, Microtus pennsylvanicus. Le sito ubi le campaniol esseva trappate esseva un ferma-lacteria con bestial cognoscitemente inficite con leptospirosis. Ben que species de Microtus es significative reservoirs de Leptospira in Russia, Europa, e altere partes del mundo, isto es le prime reporto de un isolation leptospiral ab Microtus in le Statos Unite.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave, Chicago 5, Illinois.

Bumpers for Graduated Cylinders

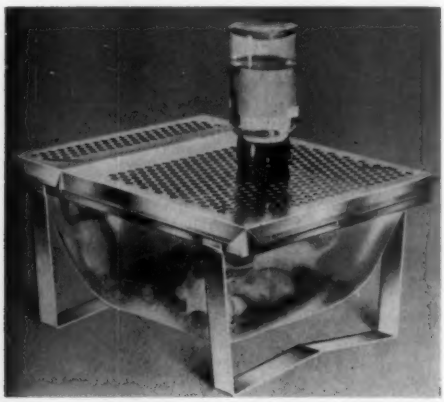
A yellow plastic ring that fits around a graduated cylinder minimizes breakage and chipping when the cylinder is tipped over. "Safe-Gard," available from



Kimble Glass Co., comes in 6 sizes to fit cylinders from 25 to 250 ml. The bumper also can be used as a visible check point for gauging volume.

Disposable Mouse Cage

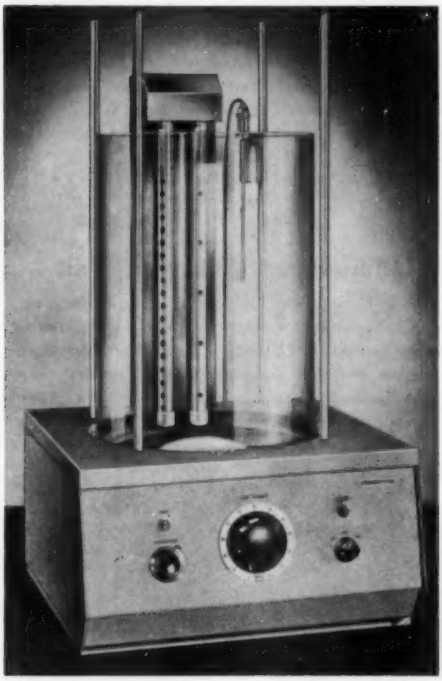
Disposable Laboratory Cages, Inc. has developed a new type of disposable mouse cage. The cage bottoms are formed from a tough, inert plastic material. The complete cage unit includes a nickel-plated steel or cardboard support, a perforated steel top, a water bottle and stainless steel tube, and a special feeding hopper.



The cage bottoms, which stack in minimum space, are transparent, permitting complete observation. Cages closely follow temperature and humidity conditions in the animal house or laboratory.

Radiant-Heat Water Bath

A water bath that uses an externally mounted 500-watt infra-red heat lamp for a heat source leaves 98% of the area in the bath free for use.



The bath, manufactured by Labline, Inc., has a temperature range of 5 to 60 C. A thermistor sensor and transistorized relay control circuit maintain uniform temperature, within ±0.005 C. A constant-speed circulating pump minimized thermal gradient, and hot spots are eliminated. The bath uses 12-inch diameter Pyrex jars, 12 or 18 inches high, with 4¾-gallon and 7½-gallon capacities, respectively.

Medical Utility Gloves

New medical utility gloves, introduced by the Pioneer Rubber Co., have a knit-cotton lining permanently bonded to a Pylox coating. The coating provides maximum flexibility and is exceptionally resistant to oils, greases, acids, and most chemicals. The gloves are 14½ in. long and have a wide cuff that can be turned back to prevent drippings from reaching upper arms.

Disposable Hospital Supplies

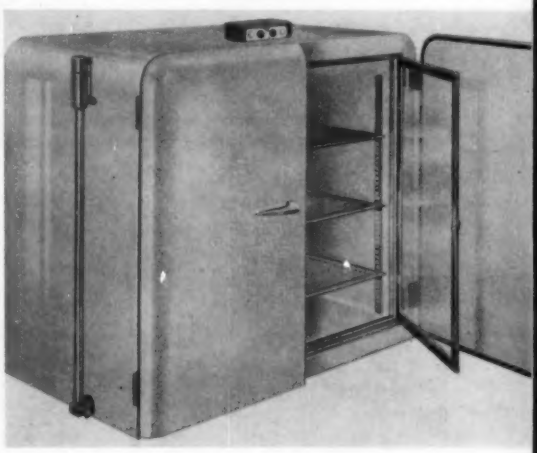
Disposable feeding bowls, carcass bags, and feces containers, costing a few cents each, are being manufactured by Busse Hospital Disposables, Inc. The 48-ounce bowls are presterilized, noiseless, and won't wilt. The disposable bag, made of laminated, water-proof creped paper, can be used for animal carcasses or in the autopsy room. It is 30 inches wide and 48 inches long. The black plastic feces container is opaque, unbreakable, leakproof, odor tight, and may be incinerated.

CO_2 Incubators

Six models of incubators, specifically designed for CO_2 incubation, are available through National Appliance Co. Continuous flow, vacuum, and batch displacement methods are used in the various models.

Temperatures are regulated and controlled by hydraulic safety and regulating thermostats with remote sensing elements. The temperature range is from room temperature to 65 C.; models giving higher temperatures are available at extra cost. (Many accessories are available for all models, and any incubator can be modified at the factory to meet individual requirements.) Differential and gradient temperatures are from ±0.3 C. to ±1.0 C.

Also available is a unit that simultaneously controls the rate of flow and the percentage of CO_2 into incubators using the continuous flow system.



Percentages from 0 to 20% and maximum flows of 16.5 liters of air-CO_2 mixed per minute can be obtained.

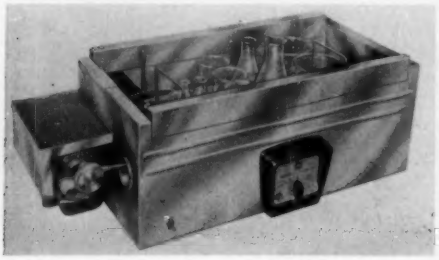
Permanent Identification

For permanent identification of animals, the Animal Identification Equipment Co. offers a kit consisting of a tattooing machine, transformer, black

marking ink, No. 2 needle bars and connecting cord. Marking can be completed in about one minute. A battery-operated power unit is also available for field use.

Shaking Bath

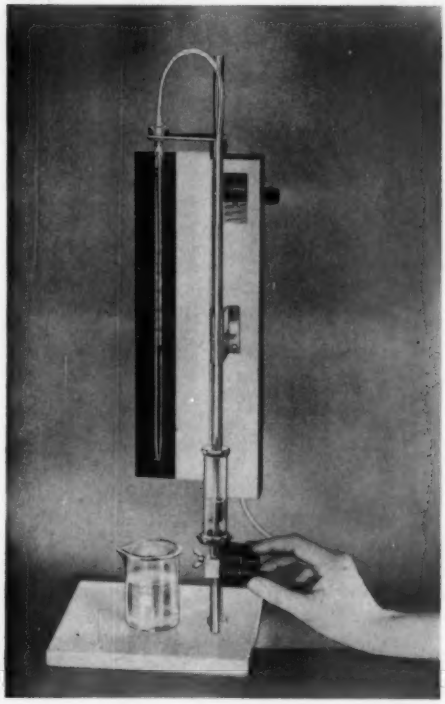
A laboratory shaking bath, designed for conditions where both continuous agitation and controlled temperatures are required, is being manufactured by Labine, Inc. The unit also may be used as a standard water bath or for shaking without water. A universal-type tray accommodates all types of glassware and test tube racks simultaneously. Stainless steel springs hold glassware firmly in place.

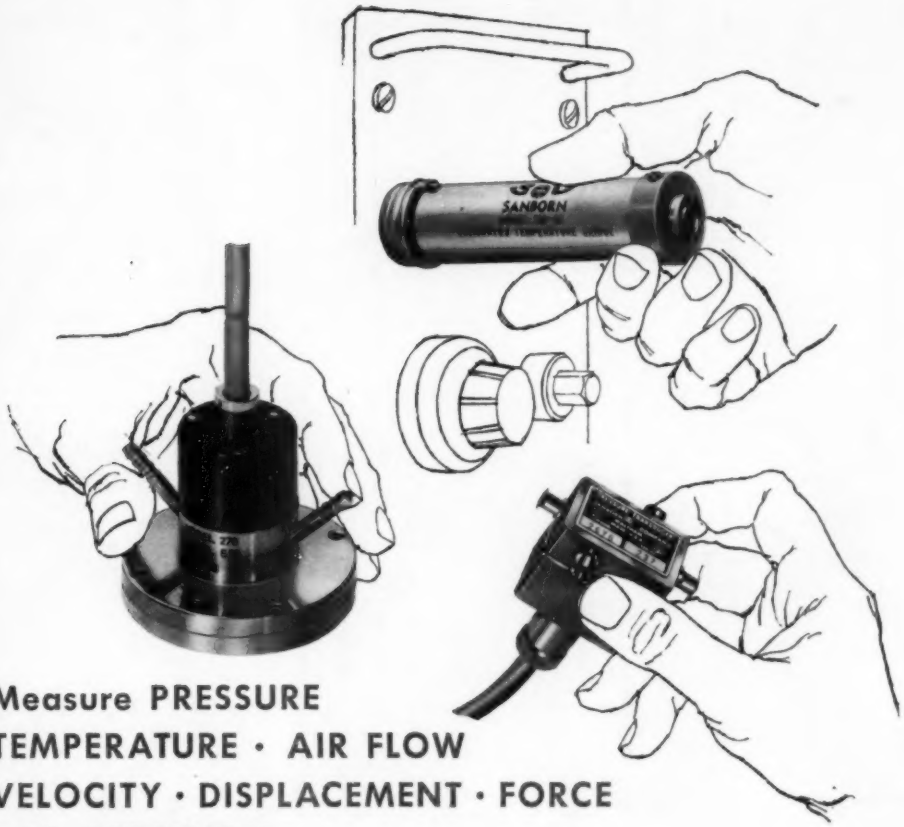


A stepless drive mechanism gives constant speed, regardless of voltage variations. Variable stroke gradations are from 0 to 2½ inches. Speeds are infinitely variable from 0 to 400 oscillations per minute. All stroke settings and speeds are reproducible. Temperature range is from 0 C. to 100 C. (32 to 212 F.) when used with hood or ice baskets. Temperature control is accurate to ± 0.5 C. More critical tolerances are also available.

Illuminated Pipetting Unit

A unit for dispensing, titrating, and decanting liquids eliminates mouth pipetting. The pipetter, manufactured by Labline, Inc., accommodates pipettes of different capacities, and dispenses quantities from 1 microliter to 20 milliliters. Light from a concealed incandescent tube is reflected on the split-color back panel, making titrant in the pipette clearly visible.





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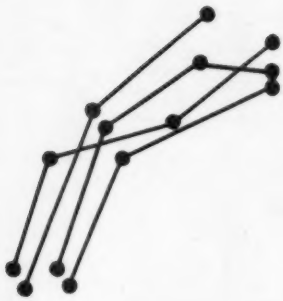

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References: 1. York, C. J.; Bittle, J. L.; Burch, G. R., and Jones, D. E.: Vet. Med. *55*:30 (April) 1960. 2. York, C. J., and Burch, G. R.: J. Am. Vet. M. A. *138*:298 (March 15) 1961.

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